Early PREdiction of Sepsis using leukocyte surface biomarkers: The ExPRES-Sepsis cohort study

Running title: Risk of sepsis using leukocyte cell surface makers

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Author contributions

Drs Shankar-Hari, Weir and Walsh had full access to all the data in the study and take responsibility for integrity of data and the accuracy of the data analyses.

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All authors confirm to the accuracy or integrity of the work.

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Conflicts of interests:

Noel Warner, Kevin Judge, Jim Keenen and Alice Wang were all employees of BD biosciences whilst this work was being undertaken, and all four authors hold stock in BD Biosciences. Prof Simpson collaborated with BDB on a Wellcome Trust/Department of Health-funded Healthcare Innovation Challenge Fund (HICF) grant in suspected ventilator-associated pneumonia. He is Director of the NIHR Newcastle In Vitro Diagnostic Evidence Co-operative (formerly the NIHR Newcastle Diagnostic Evidence Co-operative) – these entities exist to evaluate in vitro diagnostics and have worked with (and continue to work with) BDB and other companies in this capacity. All other authors declare that they do have any personal conflict of interest directly related to this manuscript.

Figures and tables; References and Supplementary material

Tables = 4; Figures = 1; References = 50; Supplementary material = 24 pages

Take home message:

In this first study of standardised multi-site flow cytometry in acutely unwell patients with suspected infections attending emergency departments, we explored which of 47 leukocyte biomarkers reliably discriminates which patients develop sepsis over the next 3 days, defined according to the Sepsis-3 sepsis criteria.

After highlighting the importance of test reliability (14 biomarkers lacked measurement reliability) and comparator cohorts (a further 17 biomarkers did not discriminate acutely unwell patients with suspected infection from patients with established sepsis-related critical illness and/or non-infective acute illness), we found that none of the remaining 16 biomarkers had clinically relevant predictive ability for subsequent sepsis or other important clinical outcomes. However, markers of early immune suppression (neutrophil and monocyte CD274 and CD279; monocyte HLA-DR) had the strongest associations with clinical outcomes. The optimum biomarker combination associated with clinical deterioration to sepsis was increased neutrophil CD24 and CD279 and reduced monocyte HLA-DR expression.

Abstract

Purpose:

Reliable biomarkers for predicting subsequent sepsis among patients with suspected acute infection are lacking. In patients presenting to emergency departments (EDs) with suspected acute infection, we aimed to evaluate the reliability and discriminant ability of 47 leukocyte biomarkers as predictors of sepsis (Sequential Organ Failure Assessment score ≥2 at 24 hours and/or 72 hours following ED presentation).

Methods:

In a multi-centre cohort study in four EDs and intensive care units (ICUs), we standardised flow-cytometric leukocyte biomarker measurement, and compared patients with suspected acute infection (cohort-1), with two comparator cohorts: ICU patients with established sepsis (cohort-2); and ED patients without infection or systemic inflammation but requiring hospitalization (cohort-3).

Results:

Between January 2014 and February 2016, we recruited 272, 59 and 75 patients to cohorts 1, 2, and 3 respectively. Of 47 leukocyte biomarkers, 14 were non-reliable, and 17 did not discriminate between the three cohorts. Discriminant analyses for predicting sepsis within cohort-1 were undertaken for eight neutrophil (cluster of differentiation antigens (CD) CD15;CD24;CD35;CD64;CD312;CD11b;CD274; CD279), seven monocyte (CD35;CD64;CD312;CD11b;HLA-DR;CD274;CD279) and a CD8 T-lymphocyte biomarker (CD279). Individually, only higher neutrophil CD279 (OR 1.78 (95%CI:1.23-2.57);P=0.002), higher monocyte CD279 (1.32 (1.03-1.70);P=0.03), and lower monocyte HLA-DR (0.73 (0.55-0.97);P=0.03) expression were associated with subsequent sepsis. With logistic regression the optimum biomarker combination was increased neutrophil CD24 and neutrophil CD279, and

reduced monocyte HLA-DR expression, but no combination had clinically relevant predictive validity.

Conclusions:

From a large panel of leukocyte biomarkers, immunosuppression biomarkers were associated with subsequent sepsis in ED patients with suspected acute infection.

Clinical trial registration: NCT02188992

Key words

Sepsis; infection; mortality; cohort study; biomarker, risk prediction

Introduction

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection[1]. Host immune responses result from leukocytes sensing pathogen- and tissue damage-associated danger signals[2, 3]. Sepsis-related immune responses involve both humoral and leukocyte components of the innate and adaptive immune systems, with excessive inflammation and immunosuppression occurring simultaneously in most patients[2, 3]. These are thought to influence the resulting clinical phenotypes and outcomes[3, 4].

Leukocyte responses in sepsis measured using flow cytometry detects leukocyte biomarkers, including surface markers and/or leukocyte subsets[5]. Previous flow cytometry-based leukocyte biomarker studies in sepsis were mostly small single centre studies in patients with sepsis, typically focusing on a limited panel of biomarkers. These studies rarely evaluated biomarker reliability and reproducibility, which is methodologically and clinically relevant as it influences diagnostic validity[6]. In addition, few studies used robust unbiased designs to assess predictive ability for clinically relevant outcomes in unselected populations with suspected infections *prior* to developing organ dysfunction and established sepsis.

We hypothesized that among patients with clinically suspected acute infection but without established sepsis, leukocyte biomarkers would identify patients who subsequently deteriorate clinically and develop sepsis, when measured within a few hours of presentation to the emergency department (ED). Our study objectives were: (1) to identify reliable leukocyte biomarkers; (2) to ascertain which of the reliable biomarkers could discriminate[6] acutely unwell patients with suspected infection from patients with community acquired sepsis-related critical illness in the intensive care unit (ICU) and/or ED patients with non-infective acute illness requiring

hospitalisation; and, (3) to ascertain whether any of the reliable biomarkers with cross-cohort discrimination could predict which patients with suspected infection in the ED subsequently develop sepsis. We also undertook a post hoc extreme phenotype analysis[7], to compare the biomarker profiles between acutely unwell patients with suspected infection who subsequently developed most severe illness with those who recovered rapidly.

Methods

Study sites and ethics

We performed a prospective, multi-centre, observational cohort study at four sites in the United Kingdom. Ethical approval was granted by the Scotland A/Oxford C Research Ethics Committees (13/SS/0023;13/SC/0266). Consent was provided by patients or surrogate decision-makers according to capacity. We registered the study (NCT02188992) and published the protocol including the analysis plan[8].

Cohort definitions and eligibility criteria

We recruited three distinct patient cohorts using an a priori sampling method to achieve similar age and sex profiles across the ED cohorts. Detailed inclusion/exclusion criteria are listed in the electronic supplement and published protocol (emethods-1)[8]. Cohort-1 comprised acutely unwell patients with suspected infection and systemic inflammation presenting to ED and formed the “discovery cohort”. Patients considered by clinical teams to already have established severe sepsis and/or require ICU admission when screened were excluded. Cohort-2 comprised ICU patients with established community acquired sepsis-related critical illness and formed the “true positive” cohort. Cohort-3 comprised acutely ill patients presenting to ED without infection or systemic inflammation but requiring

hospitalization and formed the “true negative” cohort. Inclusion criteria used throughout the study were based on the sepsis definitions by Levy et al [9], as our study was designed prior to the Sepsis-3 definitions[1, 10]. All ED patients were enrolled within 12-hours of hospital presentation. For all cohorts, we excluded patients with acute pancreatitis, haematological malignancy, chemotherapy in the past 2-weeks, myelodysplastic syndromes, known neutropenia, HIV infection, viral hepatitis infection, pregnancy, blood transfusion >4units in past week, oral corticosteroids for >24hours prior to enrolment, or a decision not for active therapy/for palliative care[8].

Leukocyte surface biomarkers and cross-site standardization of flow cytometry

We devised 5 separate flow cytometry panels to assess 47 leukocyte biomarkers with biological plausibility for having predictive validity for subsequent sepsis (eMethods-1; eTable-1; eFigure-1). We developed, standardized and harmonized flow cytometry procedures across all 4 study sites[8]. We performed flow cytometry within 4-hours of sample acquisition. All anti-human antibodies conjugated to fluorochromes for flow cytometry were from the same batch and clones (all Becton Dickinson Biosciences (BDB)), standardized on the same platform (FACSCanto II; BDB, San Jose, CA, USA), using a common batch of Cytometry Setup and Tracking beads with the same beads for daily internal quality controls, at all clinical sites. All flow cytometry standard (FCS) files were read by expert technicians using standardized gating procedures developed for each biomarker prior to analysis. The gating strategy for estimating median fluorescence intensity (MFI) or proportions is reported in eMethods-1. All FCS analysis technicians were blinded from clinical data.

Sample size

We based sample size estimates on the confidence interval (CI) widths for positive and negative predictive values (PPV and NPV). The initial design had a primary outcome of septic shock, with an estimated event rate of 5–10% in cohort-1[11, 12]. For a range in test performance for PPV/NPV of 50%-90% we planned a sample size of: cohort-1, n=300; cohort-2, n=100; and cohort-3, n=100, to give a CI width between ±4.6% to ±9.8% for PPV, and ±3.4% to ±6.3% for NPV. At an interim analysis of clinical event rates, the incidence of septic shock was substantially lower than anticipated. We decided by consensus to change the primary outcome to severe sepsis (and subsequently adopted the sepsis-3 sepsis criteria[1] of Sequential Organ Failure Assessment (SOFA) score ≥2), with critical care admission a key secondary outcome, to ensure adequate clinically relevant events in the discriminant analyses. These changes occurred *prior to* study completion and were reported in the published protocol[8].

Statistical analysis

The primary study cohort was cohort-1. The primary exposure was suspected infection. The cohorts-2 and 3 were comparator populations for cross-cohort discrimination and biomarker selection.

Outcomes

The primary outcome was sepsis, defined as SOFA score ≥2 at 24 hours and/or 72 hours following presentation to hospital in patients with suspected infection in the ED (cohort-1)[1]. Secondary outcomes were: critical care admission or death within 72 hours of presentation; SOFA ≥4 at 24 hours and/or 72 hours following presentation to hospital; development of septic shock; discharge home within 72 hours; discharge to home or in hospital with no organ failure within 72 hours; death from sepsis;

confirmed infection and length of hospital stay[8]. All cohort-1 data are based on blood samples taken in the ED after recruitment.

Biomarkers selection strategy

Our analytic approach to discover biomarkers with potential diagnostic discrimination for risk of subsequent sepsis occurred in three *a priori* planned stages, and one *post-hoc* analysis.

Stage one: reliability

Inter- and intra-reader reliability for 47 different biomarkers was established according to the protocol[8]. To be included in subsequent evaluation stages biomarkers needed to demonstrate both inter- and intra-reader reliability at the pre-defined intra-class correlation coefficient (ICC) between readers ≥0.9; see Figure-1 and eMethods-2; eTable-2). For intra-reader reliability the ICC for each reader was calculated as the ratio of within-reader variability to the total variance (within-reader plus residual variance) from the normal linear mixed model. For inter-reader reliability the ICC was calculated as the ratio of between-reader variability to the total variance (between-reader plus residual variance) from the normal linear mixed model. Reliability analyses were done prior to linking leukocyte biomarkers data and clinical outcome data.

Stage two: cross-cohort discrimination

For reliable biomarkers, statistically significant inter-group differences between the three cohorts were explored using Kruskal-Wallis analysis of variance (ANOVA) tests (eTable-3) and visual inspection of data. Biomarkers that discriminated between cohort-1 and either cohort-2 (true-positive) and/or cohort-3 (true negative) and had variability within cohort-1 consistent with potential to discriminate clinical outcomes were selected for Stage-3 analysis. Other factors considered were cell counts, the

magnitude of MFI, and potential linkage and co-linearity between groups of biomarkers. This was done in consensus meetings by researchers blinded from clinical outcomes within cohort-1.

Stage three: Prediction of clinical outcomes in cohort-1

Within cohort-1 patients, the ability of the selected biomarkers to predict the primary and secondary outcomes was calculated using univariate logistic regression. For the secondary outcomes of death from sepsis, septic shock and length of stay, we provided a descriptive summary as per the analysis plan[8]. The odds ratio (OR) for the outcome per standard deviation increase in biomarker, Receiver Operating Characteristic (ROC) curves, and area under ROC curve (AUROC) were used to assess predictive ability. Youden's index identified the optimal cut-off point for each marker[13]. Candidate biomarkers that showed consistent inclusion were then taken forward for multivariable modelling.

We used best subsets regression[14] to identify optimal combinations of predictive markers. Specifically, models containing a given number of biomarkers were fitted for all potential biomarker combinations. The five best-fitting models of a given size, according to the chi-squared score statistic, were identified. Biomarkers which consistently appeared in the best-fitting models were selected for the final model. The change in chi-squared score statistic between the best fitting models containing different numbers of biomarkers was used to determine the number of biomarkers to be included in the final model. Linearity of biomarker associations on the logistic scale was checked using plots of deviance residuals. Based on consistency and model fit we identified optimal combinations of predictive markers.

Post hoc extreme phenotype comparison

On the recommendation of a pre-planned independent expert group (see eTable-4), we compared biomarker profiles between sub-populations within cohort-1 with extreme clinical phenotypes of organ dysfunction and outcome to further explore associations for the biomarkers evaluated. We defined *well* and *sick* extreme phenotypes[7], by consensus among clinical investigators using clinical data without knowledge of group differences in biomarkers (eFigure-2). The *well* phenotype had no positive microbiology, a SOFA score ≤2 at 24 *and* 72 hours post-enrolment and were either discharged home by 72 hours or were in hospital but no longer receiving antibiotics. The *sick* phenotype had a confirmed infection, SOFA score ≥2 at both 24 *and* 72 hours post-enrolment and were still in hospital and receiving antibiotics at 72 hours. We compared biomarker expression between the two phenotypes using two-sample t-tests or Mann Whitney tests as appropriate, applying Bonferroni correction for multiple testing.

For additional comparison, we also measured C-reactive protein (CRP) and Procalcitonin (PCT) concentrations at the same time point for Cohort-1 patients, given the widespread clinical use of these biomarkers in assessing infection. We constructed ROC curves for CRP and PCT and estimated similar univariate predictive performance characteristics of these for outcomes reported, to enable direct comparison of predictive validity with the more novel biomarkers.

Results

Patient characteristics

Between January 2014 and February 2016, we recruited 272, 59 and 75 patients (N=406) to cohorts 1, 2, and 3 respectively. The clinical characteristics for the three

cohorts and the cohort-1 outcomes are shown in Table-1. Cohorts-1 and 3 had a similar age and sex distribution. Cohort-2 patients tended to be older. The primary outcome in cohort-1, clinical deterioration to sepsis, occurred in 139 patients (51.1%).

Stage one: Reliability

The step-wise assessment of intra-reader and then inter-reader reliability resulted in rejection of 14 biomarkers as non-reliable, leaving 33 reliable biomarkers for cross-cohort comparison (Figure-1; eTable-2).

Stage two: Cross-cohort discrimination

Statistical comparison, expert review, and cohort-1 data distribution resulted in rejection of a further 17 biomarkers (Figure-1; eTable-2; eTable-3). The cross-cohort comparisons plots for the 16 selected biomarkers are shown in eFigure-3. Based on the stage-1 and two selections, eight neutrophil biomarkers (cluster of differentiation antigens (CD) CD15;CD24;CD35;CD64;CD312;CD11b;CD274;CD279), seven monocyte biomarkers (CD35;CD64;CD312;CD11b;HLA-DR;CD274;CD279) and one CD8 T-lymphocyte biomarker (CD279) were selected for evaluation of discrimination for clinical outcomes. Biological relevance of these markers in sepsis are summarized in Table-2.

Stage three: Prediction of clinical outcomes in cohort-1

Most biomarkers lacked any clinically or statistically significant discrimination for predicting primary and secondary outcomes within cohort-1 patients. Amongst the individual biomarkers, clinical deterioration to sepsis was associated with higher neutrophil CD279 expression, higher monocyte CD279 expression and lower monocyte HLA-DR expression. The optimal MFI cut off for neutrophil CD279 was 239 (sensitivity 0.88 (95% confidence interval:0.82 – 0.93); specificity 0.35(0.26 –

0.43); for monocyte CD279 was 141 (sensitivity 0.83(0.77 – 0.90); specificity 0.39(0.30 – 0.47); and for monocyte HLA-DR was 3572 (sensitivity 0.43(0.34 – 0.51); specificity 0.69(0.60 – 0.77). Although these associations were statistically significant, discriminant ability was poor and unlikely to be clinically useful in isolation.

With best subsets logistic regression, the optimum combination for predicting clinical deterioration to sepsis included increased neutrophil CD24; increased neutrophil CD279; and reduced monocyte HLA-DR expression (sensitivity 0.72(0.64 – 0.79); specificity 0.56(0.48 – 0.65). With best subsets logistic regression, the optimum combination for predicting the secondary outcome of discharge to home within 72 hours, included increased neutrophil CD15, reduced neutrophil CD274 and increased total monocyte HLA-DR expression. No biomarkers had significant discriminant value for the outcome of critical care admission or death within 72 hours. The performance of individual and optimized combinations of biomarkers for predicting the primary and secondary outcomes, are shown in Table-3. No marked non-linearities in biomarker effects were identified. Overall, although statistically significant associations were demonstrated, discrimination of clinical outcomes was unlikely to be clinically useful.

Extreme phenotype analysis

From 272 patients in cohort-1, we identified 40 ‘well’ and 52 ‘sick’ phenotypes (eFigure-2). ‘Sick’ phenotype patients were characterized by being older, more often male, with a higher frequency of co-morbidities, more frequently lymphopenic, with higher APACHE II and SOFA scores at baseline. After Bonferroni correction for multiple comparisons, ‘sick’ phenotypes had significantly higher monocyte CD279

and neutrophil CD279 in the ED, but no other biomarkers were different (Table-3; eFigure-4).

For both CRP and PCT, there was also no statistically or clinically significant discrimination for subsequent sepsis with univariate analysis (Table-3).

Discussion

In this multi-site cohort study, we reduced a candidate panel of 47 leukocyte biomarkers to 16 reliable biomarkers with potential for discriminating the risk of developing sepsis in patients with suspected infection presenting to the ED. The combination of higher neutrophil CD24, higher neutrophil CD279, and a lower monocyte HLA-DR expression best predicted the clinical deterioration to sepsis. Consistent with this association, a lower neutrophil CD279 expression and higher monocyte HLA-DR expression were associated with discharge home within 72 hours (implying rapid recovery). Although our pre-defined biomarker discovery strategy identified these biomarkers as associated with development of sepsis and more severe illness, their discriminant value was insufficient to suggest utility for decision-making in routine clinical care.

Our findings have potential clinical relevance. The key pathophysiological insight is that leukocyte biomarkers of immunosuppression such as check-point inhibitors (CD279; CD274) and antigen processing ability (HLA-DR) were altered even in patients with *suspected infection* presenting to ED. We also demonstrate the importance of assessing reliability when standardising flow cytometry for large-scale time critical use. The development of clinically useable tests is likely to require a form of cross-platform calibration (such as multiparametric version of the Quantibrite system, BD Bioscience). Our study shows it is feasible to implement flow cytometry

as a means of undertaking precision medicine in sepsis, for example to guide novel therapeutic interventions such as those tested recently in immunotherapy trials [15] and highlighted in recent expert reviews [16, 17]. However, our data suggest that for patients with suspected infection the predictive validity of panels of leukocyte biomarkers are unlikely to be useful as general clinical decision-making tools. Of note, both CRP and PCT also performed poorly.

Strengths of our study were well-defined hypothesis, pre-published protocol [8], internationally accepted primary outcome [1], clinically relevant secondary outcomes and hierarchical analytic approach to reduce biomarker selection bias. Reliability of multi-site flow cytometry is potentially problematic due to measurement error bias [18], which we addressed rigorously with fluorochrome-conjugated antibody titrated for optimal signal and kept constant throughout the study. Using hospitalized non-infected patients and ICU-sepsis patients as comparators during biomarker selection increased the chance of detecting infection related host responses and is superior to using healthy volunteer controls. Our blood sampling time point in the ED was prior to severe illness, before major clinical interventions, and much earlier than in previous studies of sepsis biomarkers, and we excluded patients who clinicians considered already had established sepsis and/or critical illness. As such our population was different from other recent studies, which evaluated leukocyte biomarkers for prediction of sepsis trajectory (by including patients with sepsis-2 defined sepsis, severe sepsis and septic shock) [19, 20] and stratified nosocomial infection risk in ICU patients[21] (see eTable-5 that highlights important differences). The post-hoc extreme phenotype analysis enhanced face validity by considering multiple clinical variables simultaneously for phenotype definition.

Our study has potential weaknesses. Although we could not include all potential leukocyte biomarkers, we studied a range of leukocyte biomarkers (such as complement pathway receptors (CD35, CD11b), G protein-coupled receptors (CD312), Fc-gamma-receptors (CD64[22, 23]), factors delaying neutrophil apoptosis (CD24[22]), check-point molecules (CD274, CD279)[24]; HLA-DR expression [25-27]), that previous studies highlight association with adverse outcomes in established sepsis. We enrolled a smaller sample size than planned due to time and funding constraints. However, this had a limited impact since substantial differences in biomarker levels across cohorts still enabled selection of candidate biomarkers for further discriminant analysis. Supervised classification methods such as classification and regression trees (CART) is a valid alternative analytic approach for this research question. However, CART requires approximately 50 events per variable when predicting a dichotomised outcome, before predictions become stable and over-optimism is minimised [28]. As our observed number of sepsis events did not reach this threshold we opted to use the best subsets logistic regression approach as pre-specified in our statistical analysis plan [8]. As our cohort-1 inclusion criteria mandated SIRS, we have excluded SIRS negative patients with infection, who could have progressed to develop sepsis. However, this is unlikely to bias the results, as the prevalence of SIRS negative sepsis-3 sepsis in ICUs in England is only 3% [29]. As our objective was to study leukocyte biomarkers at an earlier time point than previously achieved and to identify biomarkers that predict deterioration within 72 hours of hospitalisation, we excluded patients planned for direct admission to ICU from the ED at enrolment, which explains the lower than expected event rate for death and septic shock. Findings might be different for more

severely ill patients studied later in sepsis, as observed in other recent flowcyometric studies (eTable-5) [19-21].

Our findings have biological plausibility, as the leukocyte biomarkers that best predicted the risk of developing sepsis in our study were on the key innate immune cells, namely neutrophils and monocytes, which are first responders to infection. The strongest biomarker predicting subsequent sepsis and extreme phenotypes was higher levels of CD279 (programmed death receptor 1, PD-1) on monocytes and neutrophils. CD279 expression is associated with neutrophil and monocyte suppressor subsets [30], memory lymphocyte subsets [31], is thought to regulate T-cell responses and induce an inhibitory signal characterized by cell cycle arrest and reduced cytokine synthesis [2, 32]. This early role for CD279/PD-1 is consistent with animal models of sepsis [33] and sepsis cohorts [30]. CD279/PD-1 act in conjunction with its ligand CD274 (PD-L1). In our study, lower CD274, together with lower CD279, higher monocyte HLA-DR, and lower neutrophil CD24, emerged as a predictor for rapid recovery sepsis phenotype. These novel findings require further confirmatory studies.

Although none of the biomarkers we studied had discriminant ability that could be used to guide clinical decision-making, our data implies that immunosuppression in infected patients precedes established sepsis and that higher CD279/PD-1 and lower HLA-DR are potential theragnostic and enrichment markers [34-37] for anti-PD-1/PDL-1 agents and granulocyte-monocyte colony stimulating factor[25] respectively, for carefully designed immunotherapy trials [3, 38].

Conclusions

We conclude that in a population of patients presenting with suspected infection, prior to established sepsis, a sequential approach to identifying reliable potential leukocyte biomarkers from a large candidate panel that may predict the subsequent development of sepsis identified only a small number with discriminant properties. These were markers of immune suppression, namely CD279 and HLA-DR, suggesting this may be an early event, prior to development of sepsis.

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TABLES

Table-1: Cohort characteristics and outcomes

	Cohort-1 (Infected ED cohort) N=272	Cohort-2 (ICU-septic) N=59	Cohort-3 (non-infected ED controls) N=75
Cohort characteristics			
Age in years mean (SD)	62.1 (19.1)	67.9 (12.8)	61.6 (20.0)
Female N (%)	133 (48.9%)	23 (39.0%)	33 (44.0%)
FCI Score Median (IQR)	2 (1,3)	2 (1,4)	1 (0,2)
White cell count Median (IQR) - **Total** - **Neutrophils** - **Lymphocytes**	 13.5 (10.7, 16.2) 11.2 (8.5, 14.1) 0.9 (0.6, 1.4)	 16.9 (10.1, 19.6) 14.1 (8.2, 17.5) 0.9 (0.6, 1.3)	 7.7 (6.4, 9.1) 4.9 (4.1, 6.4) 1.7 (1.3, 2.1)
C-reactive protein Median (IQR) **Procalcitonin Median (IQR)**	64 (21,168) 29.4 (0.0, 337.3)	212 (86,309) No data	13 (2,27) No data
Confirmed infection	238 (87.5%)	59 (100%)	0
qSOFA score >=2 - **at ED presentation** - **at 24 hours** - **at 72 hours**	 44 (16.2%) 6 (2.2%) 5 (1.8%)	 No data	 No data
APACHE II score Median (IQR)	9 (6,13)	16 (12,21)	6 (3,9)
SOFA score Median (IQR)	2 (1,3)	7 (5,9)	1 (1,2)
Site of infection N(%) - **Respiratory** - **Urinary** - **Unknown** - **Musculoskeletal, skin and soft tissue** - **Abdominal (including biliary)** - **Neurological**	 124 (45.6%) 44 (16.2%) 40 (14.7%) 32 (11.7%) 28 (11.0%) 4 (1.5%)		
Outcomes for Cohort-1			
Primary outcome[1] - **SOFA>=2 at 24 or 72 hours**	 139 (51.1%)		
Secondary outcomes - **ICU admission or death within 72 hours of hospitalization** - **SOFA>=4 at 24 or 72 hours** - **Discharged home within 72 hours of hospitalization** - **Discharged home or in hospital with no organ failure** - **Hospital mortality N (%)** - **Development of septic shock**	 22 (8.1%) 36 (13.2%) 86 (31.6%) 148 (54.4%) 1 (0.4%) 1 (0.4%)		
Organ support - **On antibiotics at 72 hours** - **Vasopressors** - **Ventilation invasive** - **Ventilation non-invasive**	 144 (52.9%) 2 (0.7%) 2 (0.7%) 5 (1.8%)		
Hospital length of stay (days) Median (IQR)	5 (2, 9)		

Table-2: Biological relevance in sepsis patients of the reliable cell surface markers with discriminant value identified in Cohort-1

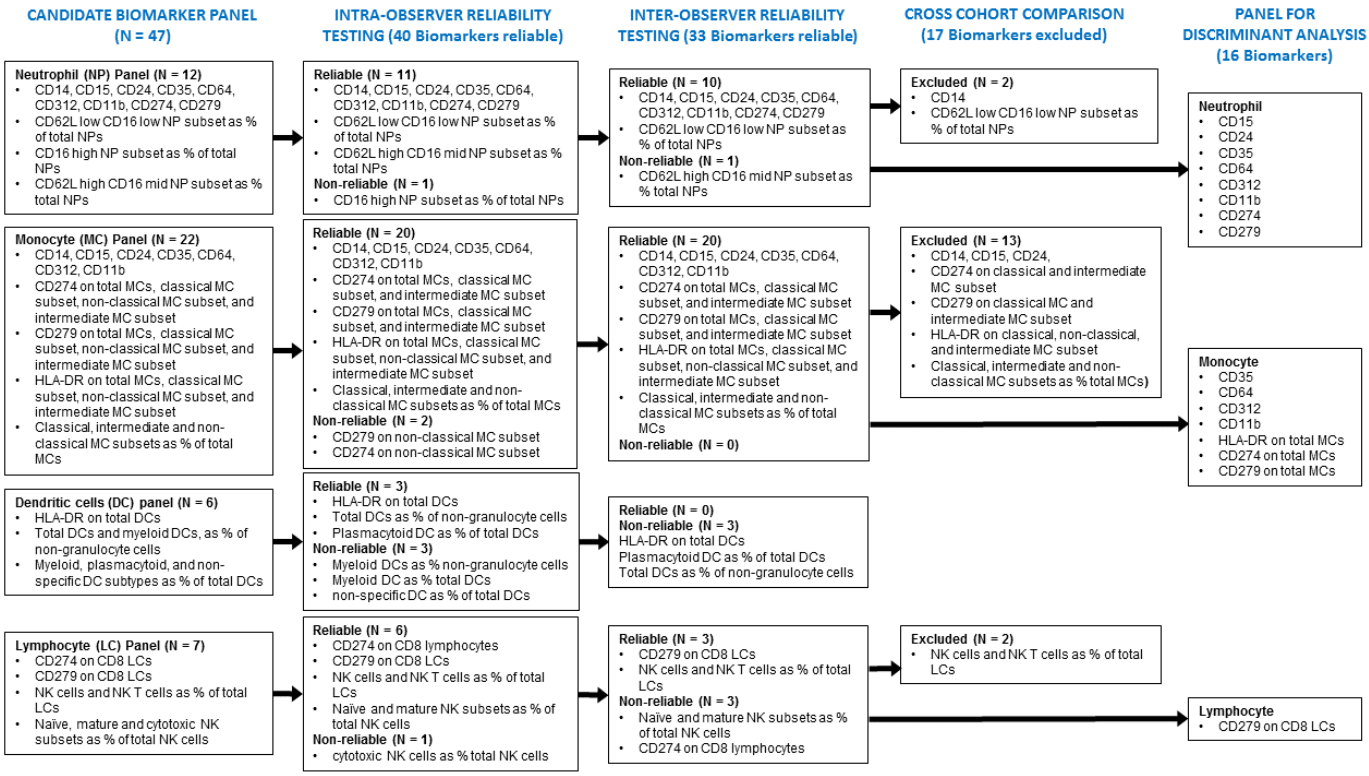
Cell surface markers	Marker positive leukocytes in our study	Biological relevance in sepsis[2, 3, 25, 32, 39-43]	Our key inferences
CD15	Neutrophil	Expressed on all myeloid cells and from the promyelocyte stage onwards on neutrophils. Although monocytes express CD15 at low levels, we were gating $CD15^{hi}$ granulocytes.	Alongside CD14, CD16, CD11b, CD15, is a marker for myeloid derived suppressor cells[44, 45], which is implicated in supressing T cell function.
CD24	Neutrophil	Expressed on mature granulocytes and B cells; down-regulated on neutrophils in sepsis, induces neutrophil apoptosis which is delayed in sepsis.	CD16 low/CD14 negative / CD24 positive myeloid-derived suppressor cells are cytotoxic to T cells[20]. Immature granulocytes in peripheral circulation in sepsis is associated with greater risk of death[46].
CD35	Neutrophil Monocytes	Receptors of complement activation (RCA) family expressed on leukocytes; potentially discriminates sepsis from inflammation	Understanding of major roles of CD35 alterations in sepsis is unclear.
CD64	Neutrophil Monocytes	Fc gamma receptor expressed on leukocytes; Patients with sepsis have increased expression of CD64 has been consistently reported.	Despite this association, CD64 as a single marker has limited diagnostic performance in sepsis [23, 41].
CD11b	Neutrophil Monocytes	Role in adhesive interactions of monocytes, macrophages and granulocytes; mediating the uptake of complement-coated particles; increased in sepsis following neutrophil activation	Neutrophil and monocyte increase in CD11b is inconsistent in the literature[47, 48]. Tissue resident CD11b positive T cells, secrete interferon gamma and may influence local host defence mechanisms in bacterial infections[49].
CD312	Neutrophil Monocytes	human myeloid-restricted class B seven-span transmembrane (TM7) subfamily of G-protein coupled receptors; acutely altered in sepsis secondary to leukocyte activation	Understanding of major roles of CD312 alterations in sepsis is unclear.
CD274	Neutrophil Monocytes	PD-1 and PDL-1 form a check point inhibitor complex and are considered markers of sepsis related immunosuppression. In sepsis, neutrophils, monocytes and lymphocytes express elevated levels of with CD274 and CD279[2, 30, 31]. In sepsis, neutrophils are thought to impair T cell function through PD-L1 mechanism[33, 50].	Recently, it has been shown that the increasing functional deficit in multiple innate and adaptive immune responses in sepsis-related critical illness could be restored ex vivo in cells treatment with monoclonal antibodies targeting either arm of the PD-1:PD-L1 axis [30, 50]. Thus, measuring cellular levels of PD-1 and PD-L1 could inform trial design.
CD279	Neutrophil Monocytes CD-8 T cells		
HLA-DR	Monocyte	Consistently reported as a marker of immunosuppression in sepsis and in critically ill patients	Reduced HLA-DR expression on monocytes is associated with increased risk of nosocomial infection due to impaired monocyte competence. Monocyte HLA-DR expression less than 8000 monoclonal antibodies/cell for 2 or more days can be reversed with GM-CSF therapy, with potentially beneficial effects [25]. This is a useful biomarker for enrichment in future clinical trials.

Table-2: Candidate biomarkers and combinations for predicting outcomes in cohort-1

Biomarker	Marker expression in cohort-1 as Median MFI (IQR)	Primary outcome [OR (95% CI) per SD increase in MFI; p value]		Secondary outcomes[1] [OR (95% CI) per SD increase in MFI; p value]				
		SOFA score ≥2 at 24 hours and/or 72 hours following presentation to hospital[2]	AUROC (95% CI)	ICU admission or death within 72 hours of presentation	SOFA>=4 at 24 or 72 hours after presentation	Discharge home within 72 hours of presentation	Discharge home within 72 hours of presentation or in-hospital with no organ failure	Confirmed infection
Neutrophils								
- CD15	31148 (22261, 39622)	0.94 (0.69 – 1.28); 0.70	0.50 (0.41 –0.59)	1.36 (0.82 – 2.22); 0.23	1.01 (0.65 – 1.58); 0.97	1.38 (0.99 – 1.91); 0.06	1.13 (0.83 – 1.56); 0.42	0.89 (0.57, 1.41); 0.63
- CD24	22261 (16398, 28565)	1.20 (0.94 – 1.54); 0.15	0.56 (0.49 – 0.63)	1.26 (0.84 – 1.90); 0.17	1.48 (1.08 – 2.05); 0.01	1.00 (0.77 – 1.30); 1.00	0.79 (0.62 – 1.02); 0.07	1.31 (0.85, 2.04); 0.22
- CD35	17363 (10021, 26452)	0.98 (0.77 – 1.25); 0.87	0.51 (0.44 – 0.58)	1.18 (0.76 – 1.83); 0.45	0.90 (0.62 – 1.31); 0.59	1.18 (0.91 – 1.53); 0.21	1.15 (0.90 – 1.47); 0.28	1.34 (0.88, 2.06); 0.17
- CD64	2384 (1353, 5522)	0.95 (0.71 – 1.29); 0.75	0.49 (0.41 – 0.58)	0.98 (0.55 – 1.75); 0.94	0.88 (0.53 – 1.45); 0.61	0.97 (0.70 – 1.33); 0.83	0.95 (0.71 – 1.28); 0.74	1.62 (0.84, 3.12); 0.15
- CD312	685 (451, 845)	1.29 (0.99 – 1.67); 0.06	0.57 (0.50 – 0.64)	0.74 (0.43 – 1.29); 0.29	0.82 (0.55 – 1.23); 0.34	0.79 (0.59 – 1.06); 0.12	0.85 (0.67 – 1.09); 0.21	1.10 (0.73, 1.67); 0.64
- CD11b	20583 (13210, 28737)	1.25 (0.97 – 1.62); 0.08	0.56 (0.49 – 0.63)	1.45 (0.97 – 2.16); 0.07	1.36 (0.98 – 1.60); 0.57	1.12 (0.86 – 1.45); 0.39	0.84 (0.66 – 1.08); 0.18	1.27 (0.83, 1.96); 0.27
- CD274	269 (207, 320)	1.25 (0.96 – 1.61); 0.10	0.57 (0.50 – 0.64)	0.91 (0.55 – 1.49); 0.70	1.16 (0.83 – 1.61); 0.39	0.70 (0.51 – 0.95); 0.02	0.77 (0.59 – 0.99); 0.045	1.56 (0.97, 2.52); 0.07
- CD279	569 (300, 640)	1.78 (1.23 – 2.57); 0.002	0.59 (0.52 – 0.66)	0.96 (0.57 – 1.61); 0.86	1.06 (0.77 – 1.46); 0.72	0.57 (0.39 – 0.83); 0.003	0.60 (0.41 – 0.87); 0.007	1.06 (0.68, 1.66); 0.78
Monocyte								
- CD35	21018 (13818, 28565)	1.15 (0.89 – 1.48); 0.28	0.55 (0.48 – 0.62)	0.99 (0.62 – 1.57); 0.95	1.33 (0.97 – 1.83); 0.07	0.91 (0.70 – 1.20); 0.52	0.94 (0.73 – 1.20); 0.60	1.19 (0.77, 1.84); 0.44
- CD64	30848 (24499, 39622)	1.04 (0.77 – 1.39); 0.80	0.57 (0.49 – 0.66)	1.25 (0.77 – 2.03); 0.36	1.12 (0.74 – 1.71); 0.59	0.95 (0.69 – 1.30); 0.73	0.92 (0.69 – 1.24); 0.58	2.24 (1.11, 4.52); 0.02
- CD312	1087 (649, 1617)	0.91 (0.71 – 1.16); 0.43	0.54 (0.47 – 0.61)	0.73 (0.41 – 1.29); 0.29	0.79 (0.52 – 1.21); 0.28	1.24 (0.96 – 1.61); 0.09	1.06 (0.83 – 1.36); 0.64	0.94 (0.65, 1.36); 0.73
- CD11b	22705 (14413, 28651)	1.21 (0.94 – 1.57); 0.14	0.58 (0.51 – 0.65)	1.25 (0.83 – 1.88); 0.28	1.27 (0.91 – 1.76); 0.16	1.15 (0.89 – 1.49); 0.30	0.87 (0.68 – 1.12); 0.27	1.24 (0.80, 1.93); 0.33
- HLA-DR	4435 (2379, 8001)	0.73 (0.55 – 0.97); 0.03	0.56 (0.49 – 0.63)	0.69 (0.34 – 1.40); 0.30	0.76 (0.46 – 1.24); 0.27	1.35 (1.04 – 1.75); 0.02	1.34 (1.00 – 1.80); 0.052	0.96 (0.66, 1.38); 0.82
- CD274	60 (0, 166)	0.90 (0.70 – 1.16); 0.41	0.50 (0.43 – 0.56)	1.06 (0.69 – 1.61); 0.80	1.03 (0.73 – 1.46); 0.85	0.84 (0.62 – 1.15); 0.28	0.99 (0.78 – 1.27); 0.95	0.89 (0.64, 1.23); 0.48
- CD279	240 (129, 280)	1.32 (1.03 – 1.70); 0.03	0.58 (0.51 – 0.65)	0.89 (0.56 – 1.43); 0.31	1.21 (0.84 – 1.75); 0.27	0.68 (0.52 – 0.90); 0.006	0.80 (0.62 – 1.02); 0.07	0.98 (0.67, 1.44); 0.92
CD8 T cells	117 (72, 169)							
- CD279		1.16 (0.81 – 1.66); 0.43	0.48 (0.41 – 0.55)	0.23 (0.02 – 2.29); 0.21	0.94 (0.58 – 1.93); 0.80	0.79 (0.43 – 1.45); 0.45	0.82 (0.55 – 1.23); 0.34	2.00 (0.44, 9.06); 0.37
Neutrophil CD24 + Neutrophil CD279		1.48 (1.10 –1.98); 0.009 2.23 (1.47 –3.38); <0.001	0.64 (0.58 – 0.71) 0.67 (0.60 – 0.74)	*	*		0.65 (0.49 – 0.87); 0.004 0.47 (0.31 – 0.71); 0.0004	*
Neutrophil CD24 + Neutrophil CD279 + Monocyte HLA-DR		1.49 (1.10 –2.00); 0.009 2.37 (1.54 –3.64); <0.001 0.72 (0.53 – 0.97); 0.03						
Neutrophil CD15 + Neutrophil CD274 + Monocyte HLA-DR						1.32 (0.94 –1.85); 0.10 0.59 (0.41 –0.86); 0.006 1.48 (1.03 – 2.13); 0.04		
Other markers#								
CRP		1.20 (0.94 – 1.54): p=0.15	0.56 (0.49, 0.63)	0.88 (0.55 – 1.42); 0.60	0.99 (0.69 – 1.42); 0.94	0.74 (0.55 – 0.99); 0.04	0.85 (0.66 – 1.08); 0.19	1.16 (0.99 – 2.65); 0.06
PCT		0.94 (0.72 – 1.21); p=0.61	0.53 (0.46, 0.60)	0.93 (0.54 – 1.61); 0.57	0.81 (0.48 – 1.36); 0.42	0.83 (0.60 – 1.15); 0.27	1.02 (0.79 – 1.32); 0.89	4.00 (0.78 – 20.5); 0.10

Table-4: Extreme phenotype description

	Well phenotype (N = 40)	**Sick phenotype (N = 52)**	**p-value**
Age, median (IQR)	37.5 (27.3 – 56.8)	70.0 (56.0 – 81.0)	<0.001
Female, n (%)	26 (65%)	19 (37%)	0.009
FCI Score Median (IQR)	1 (0 – 2)	2 (1 – 3)	0.03
White cell count Median (IQR)			
Total	13.2 (10.3 – 14.4)	13.1 (9.1 – 16.5)	0.78
Neutrophils	10.3 (8.1 – 12.0)	11.2 (7.5 – 15.1)	0.29
Lymphocytes	1.2 (0.7 – 1.8)	0.8 (0.5 – 1.2)	0.01
C-reactive protein Median (IQR)	58.5 (24.0 – 107.3)	56.0 (16.5 – 191.0)	0.85
qSOFA score >=2			
at ED presentation	3 (7.5%)	10 (19.2%)	0.11
at 24 hours	0	4 (7.7%)	0.07
at 72 hours	0	2 (3.8%)	0.22
Source of infection*, n (%)			
Respiratory	13 (40.6 %)	30 (57.7%)	0.13
Neurological	1 (3.1%)	2 (3.8%)	0.87
Urinary	2 (6.3 %)	7 (13.4%)	0.31
Abdominal	5 (15.6 %)	5 (9.6%)	0.41
Skin	9 (28.1 %)	3 (5.8%)	0.005
Biliary	0 (0%)	5 (9.6%)	0.005
Sepsis of unknown origin	2 (6.3 %)	0 (0%)	0.07
Baseline APACHE 2 score, median (IQR)	4.5 (2-7)	11.5 (9-16)	<0.001
Baseline SOFA, median (IQR)	1 (1-1)	3 (2-4)	<0.001
Discharged home within 72 hours, n (%)	32 (80%)	0	<0.001
Admitted to HDU/ICU within 72 hours, n (%)	0	14 (26.9%)	<0.001
Neutrophil biomarkers (MFI) median (IQR)			
Neutrophil CD15	30848 (24499 – 45352)	30848 (19116 – 41992)	>0.10
Neutrophil CD24	23815 (18299 – 29261)	24034 (18741 – 30710)	>0.10
Neutrophil CD35	19485 (7985 – 26580)	15636 (10988 – 25117)	>0.10
Neutrophil CD64	3098 (1528 – 6272)	2150 (1693 – 5378)	>0.10
Neutrophil CD312	565.8 (382.7 – 712.9)	670.9 (493.6 – 853.9)	>0.10
Neutrophil CD11b	16089 (13664 – 25552)	22154 (13510 – 30737)	>0.10
Neutrophil CD27s	279.0 (101.4 – 322.8)	284.3 (233.8 – 327.7)	>0.10
Neutrophil CD279	326.4 (152.7 – 584.2)	584.2 (383.7 – 648.8)	**0.005**
Monocyte biomarkers (MFI) median (IQR)			
Monocyte CD35	16556 (9974 – 27488)	22476 (15067 – 27681)	>0.10
Monocyte CD64	29685 (21843 – 45021)	33323 (29405 – 45352)	>0.10
Monocyte CD312	1243 (694 – 2001)	817.0 (470.5 – 1560.0)	>0.10
Monocyte CD11b	20205 (12102 – 26644)	26660 (16984 – 32741)	>0.10
Monocyte CD274	50.7 (0 – 167.2)	78.6 (0 – 199.7)	>0.10
Monocyte CD279	151.2 (94.8 – 262.1)	245.4 (161.1 – 287.0)	**0.05**
Monocyte HLA-DR	6172 (3516 – 11544)	4016 (2692 – 7170)	0.12
CD-8 T cell biomarker (MFI) median (IQR)			
CD8 T-Lymphocyte CD279	112.2 (78.7 – 153.3)	115.6 (58.5 – 167.9)	>0.10


CANDIDATE BIOMARKER PANEL (N = 47)
INTRA-OBSERVER RELIABILITY TESTING (40 Biomarkers reliable)
INTER-OBSERVER RELIABILITY TESTING (33 Biomarkers reliable)
CROSS COHORT COMPARISON (17 Biomarkers excluded)
PANEL FOR DISCRIMINANT ANALYSIS (16 Biomarkers)
Neutrophil (NP) Panel (N = 12)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b, CD274, CD279
CD62L low CD16 low NP subset as % of total NPs
CD16 high NP subset as % of total NPs
CD62L high CD16 mid NP subset as % total NPs
Reliable (N = 11)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b, CD274, CD279
CD62L low CD16 low NP subset as % of total NPs
CD62L high CD16 mid NP subset as % total NPs
Non-reliable (N = 1)
CD16 high NP subset as % of total NPs
Reliable (N = 10)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b, CD274, CD279
CD62L low CD16 low NP subset as % of total NPs
Non-reliable (N = 1)
CD62L high CD16 mid NP subset as % total NPs
Excluded (N = 2)
CD14
CD62L low CD16 low NP subset as % of total NPs
Neutrophil
CD15
CD24
CD35
CD64
CD312
CD11b
CD274
CD279
Monocyte (MC) Panel (N = 22)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b
CD274 on total MCs, classical MC subset, non-classical MC subset, and intermediate MC subset
CD279 on total MCs, classical MC subset, non-classical MC subset, and intermediate MC subset
HLA-DR on total MCs, classical MC subset, non-classical MC subset, and intermediate MC subset
Classical, intermediate and non-classical MC subsets as % of total MCs
Reliable (N = 20)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b
CD274 on total MCs, classical MC subset, and intermediate MC subset
CD279 on total MCs, classical MC subset, and intermediate MC subset
HLA-DR on total MCs, classical MC subset, non-classical MC subset, and intermediate MC subset
Classical, intermediate and non-classical MC subsets as % of total MCs
Non-reliable (N = 2)
CD279 on non-classical MC subset
CD274 on non-classical MC subset
Reliable (N = 20)
CD14, CD15, CD24, CD35, CD64, CD312, CD11b
CD274 on total MCs, classical MC subset, and intermediate MC subset
CD279 on total MCs, classical MC subset, and intermediate MC subset
HLA-DR on total MCs, classical MC subset, non-classical MC subset, and intermediate MC subset
Classical, intermediate and non-classical MC subsets as % of total MCs
Non-reliable (N = 0)
Excluded (N = 13)
CD14, CD15, CD24,
CD274 on classical and intermediate MC subset
CD279 on classical MC and intermediate MC subset
HLA-DR on classical, non-classical, and intermediate MC subset
Classical, intermediate and non-classical MC subsets as % total MCs)
Monocyte
CD35
CD64
CD312
CD11b
HLA-DR on total MCs
CD274 on total MCs
CD279 on total MCs
Dendritic cells (DC) panel (N = 6)
HLA-DR on total DCs
Total DCs and myeloid DCs, as % of non-granulocyte cells
Myeloid, plasmacytoid, and non-specific DC subtypes as % of total DCs
Reliable (N = 3)
HLA-DR on total DCs
Total DCs as % of non-granulocyte cells
Plasmacytoid DC as % of total DCs
Non-reliable (N = 3)
Myeloid DCs as % non-granulocyte cells
Myeloid DC as % total DCs
non-specific DC as % of total DCs
Reliable (N = 0)
Non-reliable (N = 3)
HLA-DR on total DCs
Plasmacytoid DC as % of total DCs
Total DCs as % of non-granulocyte cells
Lymphocyte (LC) Panel (N = 7)
CD274 on CD8 LCs
CD279 on CD8 LCs
NK cells and NK T cells as % of total LCs
Naïve, mature and cytotoxic NK subsets as % of total NK cells
Reliable (N = 6)
CD274 on CD8 lymphocytes
CD279 on CD8 LCs
NK cells and NK T cells as % of total LCs
Naïve and mature NK subsets as % of total NK cells
Non-reliable (N = 1)
cytotoxic NK cells as % total NK cells
Reliable (N = 3)
CD279 on CD8 LCs
NK cells and NK T cells as % of total LCs
Non-reliable (N = 3)
Naïve and mature NK subsets as % of total NK cells
CD274 on CD8 lymphocytes
Excluded (N = 2)
NK cells and NK T cells as % of total LCs
Lymphocyte
CD279 on CD8 LCs

Online only supplement

Shankar-Hari M et al. Early PREdiction of Sepsis using leucocyte cell surface biomarkers: The (ExPRES-Sepsis) cohort study

eMethods

eMethods-1: Detailed study cohort description[1] and description of gating with rationale
eMethods-2: Reliability and optimisation

eTables

eTable-1: Leukocyte biomarkers evaluated
MFI = mean fluorescence index; CD = Cluster of differentiation;

eTable-2: Table of Intraclass correlation coefficients (ICC) from the intra- and inter-rater reliability studies for each of the 47 biomarkers. Biomarkers rejected on intra-rater reliability testing are shown in red. Biomarkers rejected on inter-rater reliability testing are shown in blue

eTable-3: Rationale for Biomarkers selected for discriminant analysis in cross cohort comparison

eTable-4: Members of the independent expert review group who reviewed the provisional data from reliability, cross cohort comparisons, and discriminant analysis for the primary and secondary outcomes.

eTable-5: Comparison of recent leukocyte biomarker studies using multi-site flow cytometry with standardisation for illness trajectory prediction [2-4]

eFigures

eFigure-1: Flow diagram showing the decision analysis for assessing intra- and inter-rater reliability for the 47 biomarkers, and selecting biomarkers considered reliable for evaluation in cross cohort comparisons, as reported in the published protocol.

eFigure-2: Extreme phenotype derivation algorithm

eFigure-3: Cross cohort comparison of significant markers taken forward for further evaluation

eFigure-4: Comparison of biomarkers between sick phenotype, and well phenotypes
Shows significant differences in neutrophil CD279 between sick and well phenotype. MFI = Median Fluorescence intensity, reported on log10 scale. Statistical significance was determined using the Bonferroni-Dunn method to correct for multiple testing, with alpha = 0.05.

eMethods-1: Detailed study cohort description

This was reported in the published protocol paper and is replicated here for completion[1].
We recruited three distinct patient cohorts:
Cohort 1: Patients presenting to hospital with suspected infection with a systemic inflammation (discovery cohort).
Cohort 2: hospitalised patients with community-acquired severe sepsis requiring treatment in critical care (true- positive cohort).
Cohort 3: patients presenting with no suspicion of infection or systemic inflammation, needing hospitalisation ('non-sepsis comparison population').

Inclusion criteria

Cohort 1
Age ≥16 years (≥18 years in England), (2) SIRS criteria met, (3) clinical suspicion of sepsis (blood cultures and/ or other samples taken for microbial culture, or antibiotics started by clinical team), (4) no clinical suspicion of severe sepsis or septic shock at the time of enrolment and (5) enrolled within 12hours of hospital (ED) presentation.
Cohort 2
Age ≥16 years (≥18 years in England), (2) SIRS criteria met, (3) clinical suspicion of sepsis (blood cultures and/ or other samples taken for microbial culture, or antibiotics started by clinical team), (4) severity of sepsis requiring critical care admission (based on decision of caring clinical teams), (5) enrolled within 72 hours of hospital admission and (6) not enrolled into cohort 1 of ExPRES-Sepsis.
Cohort 3
Age ≥16 years (≥18 years in England), (2) does not meet SIRS criteria, (3) no clinical suspicion of sepsis (blood cultures and/or other samples NOT taken for microbial culture, and antibiotics NOT started by clinical team), (4) patient expected to be admitted to hospital, (5) patient NOT expected to die during hospital admission.

Exclusion criteria (for all cohorts)

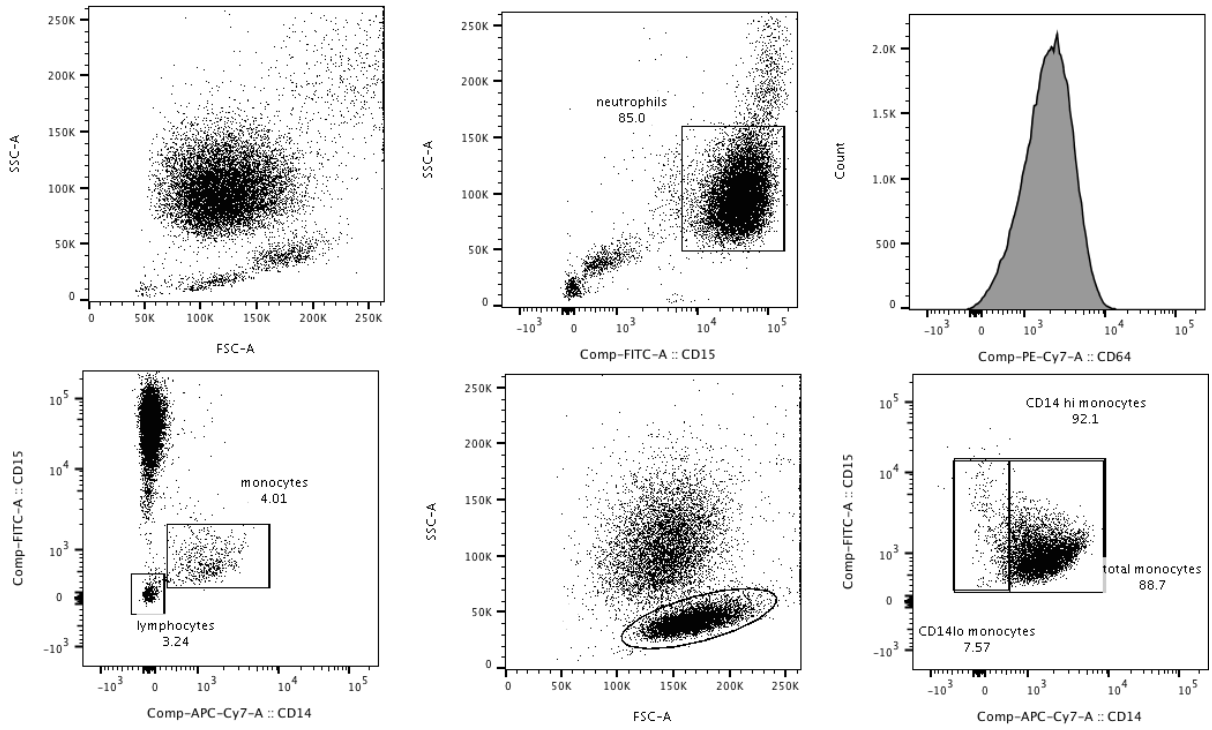
Exclusions were chosen to ensure conditions that provoke a sterile inflammatory response or lead to immune dysfunction did not act as confounders during flow cytometry analysis. Patients who would not be actively treated were also excluded.
The exclusion criteria are any of: (1) acute pancreatitis, (2) haematological malignancy, (3) recent chemo- therapy (past 2 weeks), (4) myelodysplastic syndromes, (5) known neutropenia, (6) HIV infection, (7) viral hepatitis infection, (8) pregnancy, (9) blood transfusion >4units in past week, (10) oral corticosteroids for >24hours prior to enrolment, (11) decision not for active therapy/for palliative care at admission and (12) inability to consent the patient.

eMethods-1: Description of gating and rationale

An a priori standard operating gating procedure was developed to identify other leukocyte biomarkers using the raw flow cytometry data. This involved: (1) initial strategy, based on pre-existing data, (2) expert learning and strategy refinement, to ensure ideal identification of leukocyte subtypes, and (3) expert consensus and finalisation of the gating strategy. These stages were undertaken iteratively by expert flow cytometrists (at least 2 years of flow cytometry experience) with cycles of testing and re-testing until a final procedure for each biomarker was agreed. We designed five separate panels.

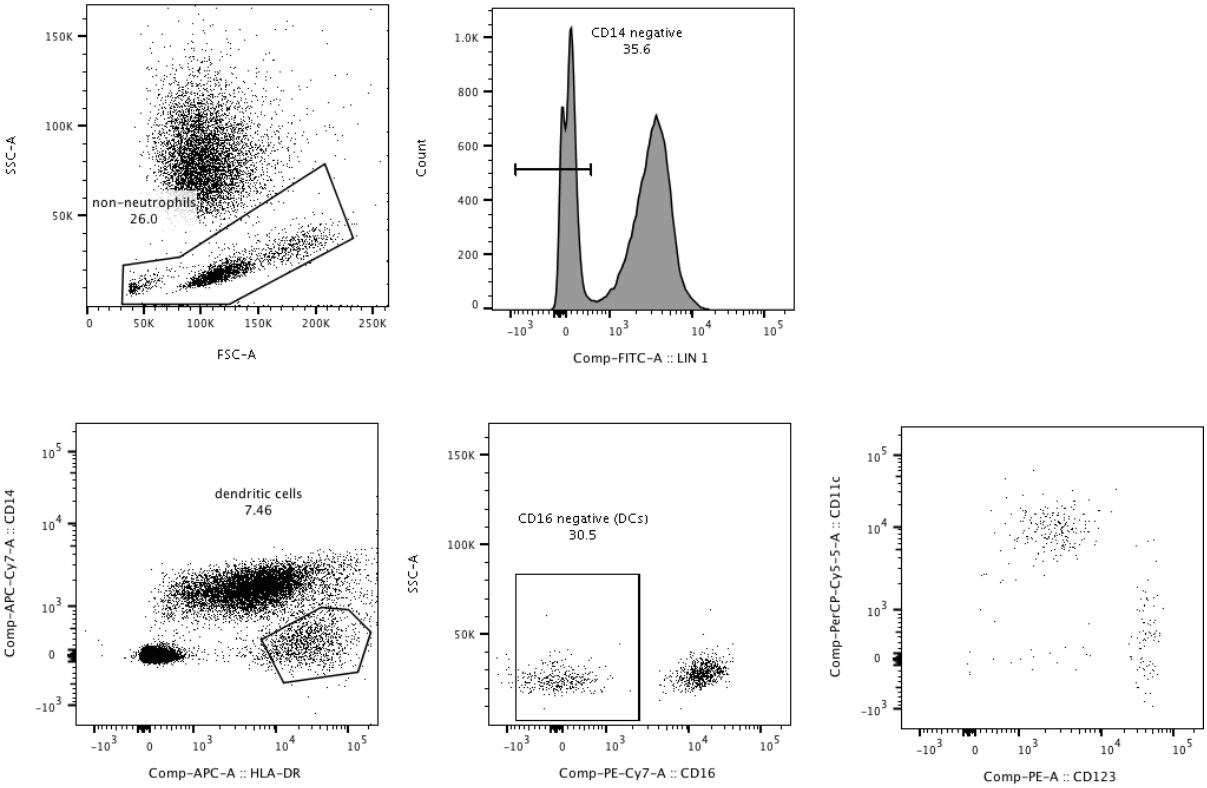
Panel-A

Marker	Fluorophore	Clone	Rationale/justification for use in the study
CD14	APC-H7	MΦP9	CD14 and CD15 have been chosen to help isolate monocytes and neutrophils. CD24, CD35, CD64 and CD312 have been chosen as markers of sepsis. Neutrophil CD64 is increased in infections. CD24 has been noted to be up regulated in in-vitro models of sepsis and blocking this pathway has been suggested to ameliorate sepsis. An increased CD312 (EMR2) expression on neutrophils has been liked with SIRS. An increase in CD35 expression has been linked to bacterial infection as compared to viral infection.
CD15	FITC	W6D3	
CD24	PerCP-Cy5.5	ML5	
CD35	PE	E11	
CD64	PE-Cy7	10.1	
CD312	AF647 (APC)	2A1 Serotec	



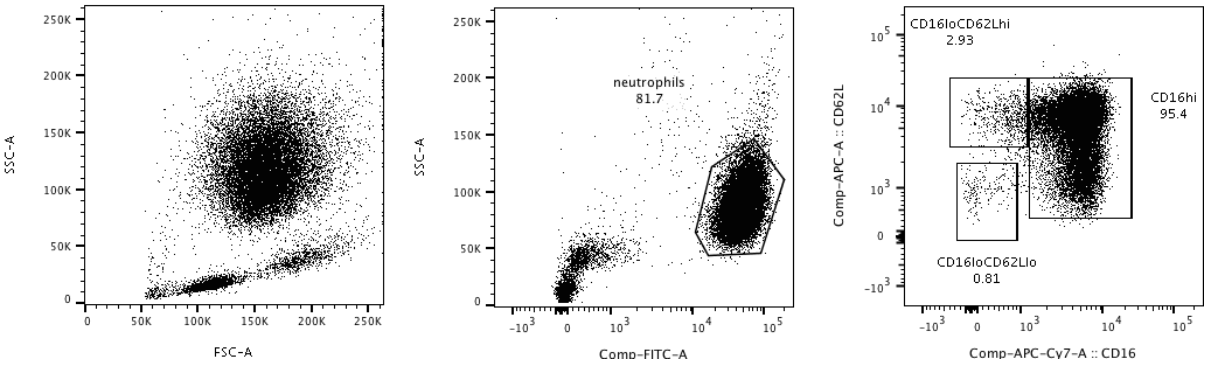
Panel B

Marker	Fluorophore	Clone	Rationale/justification for use in the study
CD3	FITC	SK7	The strategy at looking at myeloid and plasmacytoid dendritic cells is based on previous studies and a commercial assay developed by BD Biosciences. CD3, CD19 and CD56 have been chosen, all on the FITC channel, as lineage selection markers to help gate for dendritic cells. CD11c and CD123 have been chosen to allow differentiation between dendritic cell subtypes. CD14 and CD16 have been chosen to detect monocyte subtypes. Low monocyte HLA-DR has been associated with poor outcome in sepsis.
CD11c	PerCP-Cy5.5	S.HCL-3	
CD14	APC-H7	MΦP9	
CD16	PE-Cy7	B73.1	
CD19	FITC	4G7	
CD56	FITC	NCAM16.2	
CD123	PE	9F5	
HLA-DR	APC	G46-6	



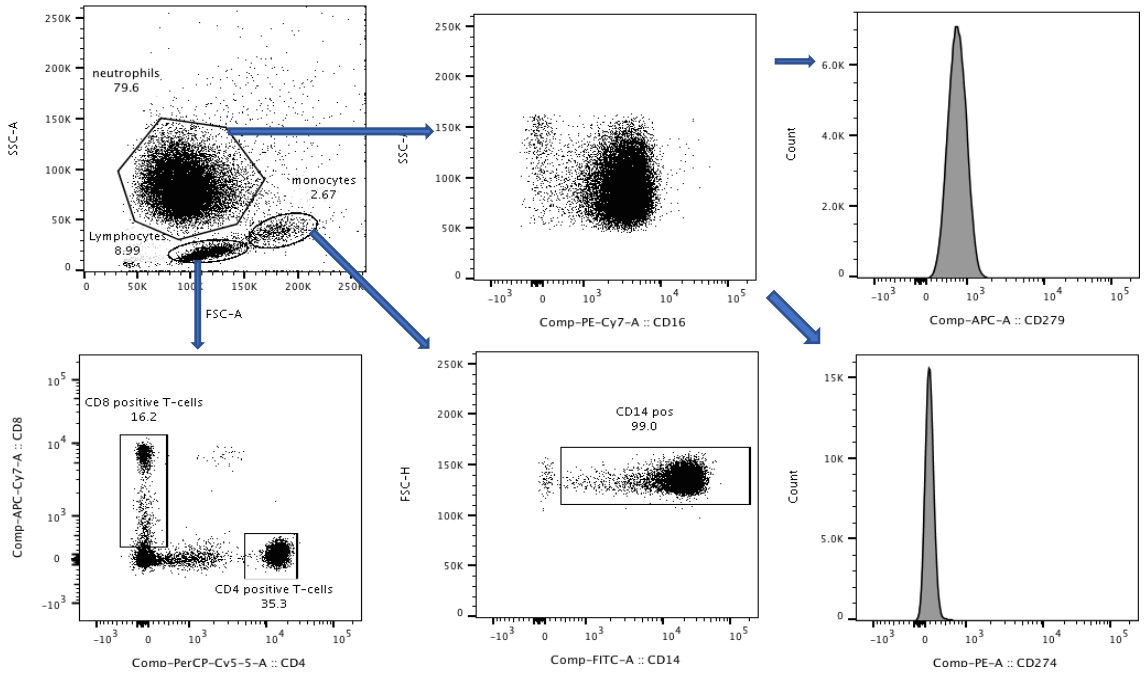
Panel-C

Marker	Fluorophore	Clone	Rationale/justification for use in the study
CD9	PE	M-L13	CD11b and CD62L have been chosen to be investigated as markers of sepsis. CD11b expression is enhanced in neonatal sepsis. CD9, CD15 and CD16 have been chosen to explore neutrophil progenitors, as immature neutrophils are associated with worse outcomes in sepsis patients.
CD11b	PE-Cy7	ICRF 44	
CD15	FITC	W6D3	
CD16	APC-H7	3G8	
CD62L	APC	DREG-56	



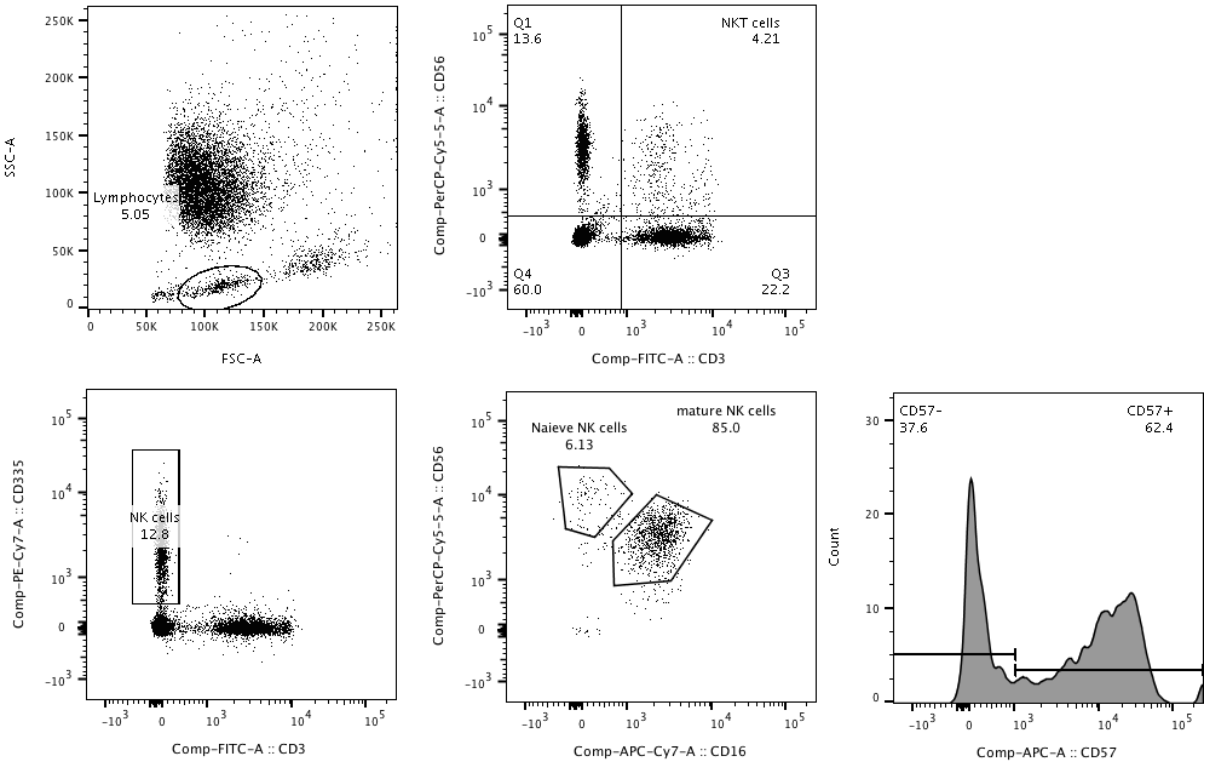
Panel-D

Marker	Fluorophore	Clone	Rationale/justification for use in the study
CD4	PerCp-Cy5.5	SK3	CD4 and CD8 have been chosen to differentiate between T-helper and cytotoxic T cells. CD14 and CD16 have been chosen to detect monocyte subtypes. CD274 (PD-L1) and CD279 (PD-1) are being assessed as potential markers of predicting sepsis, as increased expression is associated with worse outcomes in critically ill sepsis cohorts.
CD8	APC-H7	SK1	
CD14	FITC	MΦP9	
CD16	PE-Cy7	B73.1	
CD274	PE	MIH1	
CD279	APC	MIH4	



Panel-E

Marker	Fluorophore	Clone	Rationale/justification for use in the study
CD3	FITC	SK7	CD3 detects lymphocyte, NK and NKT cell populations. CD56 has been chosen to detect NKT cells. CD335 has been chosen to detect NK cells. CD16, CD56 and CD57 have been chosen to detect NK cell subsets.
CD16	APC-H7	3G8	
CD56	PerCP-Cy5.5	B159	
CD57	APC	NK-1	
CD284	PE	T901	
CD335	PE-Cy7	p9E2/NKp46	



Gating procedure

We evaluated stability of sample acquisition assessment by plotting time versus forward scatter and eliminated sections of poor flow. We then excluded cell aggregates using flow height versus area on the forward scatter. Further artefacts (debris) were eliminated by creating an “all cells” gate on the forward versus side scatter. Bi-exponential scaling was used for displaying and gating of flow cytometry data.

Neutrophil biomarkers

1. Display CD15 vs SSC on the ‘all cells’ gate for granulocytes
2. Gate tightly on $CD15^{hi}$ for neutrophils
3. Determine MFI for true neutrophil population for CD14, CD15, CD24, CD35, CD64, and CD312

Monocyte biomarkers

1. Display CD14 vs CD15 on all cells gate
2. Gate lymphocytes as $CD14^{lo}$ / $CD15^{lo}$
3. Display FSC-A vs SSC-A on all cells gate
4. Gate for presumed monocytes by light scatter properties
5. Calculate true monocyte gate as “presumed monocytes” NOT lymphocytes NOT neutrophils
6. Display CD14 vs CD15 on true monocyte gate

7. Plot 3 gates: total monocytes will be the largest rectangular gate, and 2 further gates: $CD14^{hi}$ monocytes and $CD14^{lo}$ monocytes
8. Determine MFI for CD14, CD15, CD24, CD35, CD64, and CD312 markers on each monocyte population

Dendritic cells

1. Display Lin^- (FITC-A) histogram on non-neutrophil gate (Note when referring to Lin- (lineage negative) we are referring to the $CD3^{negative}$ / $CD19^{negative}$ / $CD56^{negative}$ population.
2. Gate for Lin^- population using the marker tool - take the upper marker to the base of the Lin^+ population
3. Display HLA-DR vs CD14 on the above Lin^- subpopulation
4. Gate $HLA\text{-}DR^{positive}$ / $CD14^{negative}$ sub-population using a square or polygon gate
5. Display sub-population as CD16 vs SSC
6. Gate for total dendritic cells (DCs) as $CD16^{negative}$ using a square gate
7. Report total DCs as % of non-granulocyte cells
8. Report MFI of HLA-DR on total DCs

Dendritic cell subsets

1. Display CD123 vs CD11c on the above total DCs
2. Gate for myeloid dendritic cells as $CD11c^{high}$ / $CD123^{low}$
3. Gate for plasmacytoid dendritic cells as $CD11c^{low}$ / $CD123^{high}$
4. Gate for non-specific dendritic cells as $CD11c^{low}$ / $CD123^{low}$
5. Report DC subtypes as % of total DCs
6. Report mDC as % of non-granulocytes

Neutrophil progenitors

1. Display neutrophil population for CD16 vs CD62L
2. Gate the following neutrophil sub-types:
    a. $CD16^{hi}$ as mature neutrophils
    b. $CD62L^{hi}$ / $CD16^{mid}$ (as presumed late immature neutrophils)
    c. $CD62L^{lo}$ / $CD16^{lo}$ (as early immature neutrophils)
3. Record each gate as percentage of total granulocytes

Natural Killer T cells

1. Display CD3 vs CD56, on total lymphocytes
2. Report NKT cells (CD3+/CD56+) as % of total lymphocytes

Natural Killer cells and subsets

1. Display CD3 vs CD335, on total lymphocytes
2. Gate NK cells as $CD3^-/CD335^+$
3. Report total NK cells as % of total lymphocytes
4. NK subsets
    a. Display CD16 vs CD56 on total NK cells
    b. Gate naïve NK subset as $CD56^{++}$ / $CD16^-$; report as % of total NK cells
    c. Gate mature NK subset as $CD56^+$ / $CD16^+$; report as % of total NK cells
    d. Cytotoxic NK subset
        i. Display histogram of CD57 on $CD56^+$ / CD16++ mature NK subset (from previous step)
        ii. Report $CD57^{high}$ cytotoxic NK subset as % of mature NK cells (i.e. parent population)

eMethods-2: Reliability and optimisation

Sample size

Based on published recommendations[5], a sample size of 50 files was selected for the measurement of inter-observer agreement, and 13 files were selected for intra-observer agreement. For intra-observer agreement each expert observer re-analysed a different set of 13 files. For inter-observer agreement three different readers analysed the same 50 files. All 47 biomarkers were read from each file. For the intra-observer agreement, the files were presented in random order to readers by an independent individual.

Statistical testing of reproducibility

Each panel biomarker was assessed separately for intra- followed by inter-rater reliability. Reliability was assessed using intra-class correlation (ICC) coefficients and Bland-Altman plots. These measures were generated for both intra- and inter- observer agreement. ICC is expressed on a scale from 0 to 1: an ICC of 1.0 is interpreted as no variance between each observer, the ideal situation where observers can be considered interchangeable. An ICC cut-off of 0.9 was selected as the threshold for selection on the basis of previous literature. ICCs below this were judged to have inadequate reliability.

Analysis of each laboratory marker generated 3 intra-observer ICC coefficient statistics, describing the repeatability of reading by each observer. For inter-rater reliability each marker had 1 ICC coefficient statistic, describing the repeatability of the gating strategy across the 3 observers. Further assessment of each reader and each marker was performed using descriptive summary statistics and Bland-Altman statistics as required. Logarithmic transformation of data was carried out for further analyses if any data was not normally distributed. Bland-Altman plots were displayed as means vs differences, with the mean of differences referred to as bias, and upper and lower limits of agreements (U-LoA and L-LoA respectively) also generated.

Interpretation of reliability statistics

A protocol and rules-based system was created *a priori* to interpret the results of the reliability study (Figure 1). Intra-observer reliability was assessed first; the rationale being that intra-rater reliability was essential before any comparison between readers was likely to have clinical utility in discriminant analysis. If intra-rater reliability was established, the analysis proceeded to inter-observer reliability analysis. We required biomarkers to demonstrate both intra- and inter-rater reliability in order to be taken to the cross-cohort comparisons.

The interpretation strategy was designed to allow re-examination of markers which might potentially be falsely excluded due to any single data points which might be outliers.

Protocol for reliability assessment

Intra-observer reliability

Step 1

a. Is the intra-observer ICC for all 3 observers is greater than or equal to 0.9?
b. If yes, the outcome will be classified as having high reliability, and biomarker taken forward to the inter-observer interpretation stage
c. If no, proceed to step 2

Step 2

a. Do 2 out of 3 observers have an intra-observer ICC of greater than or equal to 0.9?
b. If no, outcome classified as having low reliability, and biomarker not be taken forward for primary analysis
c. If yes, proceed to step 3

Step 3

a. Data further analysed with a Bland-Altman plot and summary statistics. Qualitative and quantitative assessment of systematic bias, limits of agreement, and note if there are any points felt to be outliers.
b. Expert decision whether biomarker should be re-classified as having high reliability?
c. If yes, biomarker taken forward to the inter-observer interpretation stage
d. If no, biomarker not taken forward

Inter-observer reliability data

Step 1

a. Is the overall inter-observer ICC greater than or equal to 0.9?
b. If yes, the biomarker classified as having high reliability.
c. If no, proceed to step 2.

Step 2

a. Was poor agreement caused by a small number of outlier comparisons from one reader?
b. Was poor agreement attributable due to a single reader with a systematic bias?
c. Were limits of agreement consistent with acceptable precision?
d. Based on a-c assessment biomarker classified as having adequate reliability or rejected from further evaluation.

Results from statistical assessment

The results from the intra- and inter-rater reliability studies is shown in etable-2.

eTable-1: Leukocyte biomarkers evaluated

Leukocyte subset	Biomarker measurement description
Neutrophil biomarkers (N = 12 biomarkers)	MFI for CD14, CD15, CD24, CD35, CD64, CD312, CD11b, CD274, CD279
	CD62L low CD16 low neutrophil subset as proportion of total neutrophils
	CD16 high neutrophil subset as proportion of total neutrophils
	CD62L high CD16 mid neutrophil subset as proportion of total neutrophils
Monocyte biomarkers (N = 22 biomarkers)	MFI for CD14, CD15, CD24, CD35, CD64, CD312, CD11b
	MFI for HLA-DR as measured on total monocytes, classical monocyte subset, non-classical monocyte subset, and intermediate monocyte subset
	MFI for CD274 as measured on total monocytes, classical monocyte subset, non-classical monocyte subset, and intermediate monocyte subset
	MFI for CD279 as measured on total monocytes, classical monocyte subset, non-classical monocyte subset, and intermediate monocyte subset
	Classical, intermediate and non-classical monocyte subsets, all 3 as proportion of total monocytes
Dendritic cell biomarkers (N = 6 biomarkers)	MFI for HLA-DR measured on total dendritic cells
	Total dendritic cells, and myeloid dendritic cells, both as proportion of non-granulocyte cells
	Myeloid, plasmacytoid, and non-specific dendritic cell subtypes, as proportion of total dendritic cells
Lymphocyte biomarkers (N = 2 biomarkers)	MFI for CD274 and CD279 measured on CD8 lymphocytes
Natural killer cell biomarkers (N = 5 biomarkers)	Natural Killer cells and NKT cells, both measured as proportion of total lymphocytes
	Naïve, mature and cytotoxic NK subsets, measured as proportion of total Natural Killer cells

eTable-2: Table of Intraclass correlation coefficients (ICC) from the intra- and inter-rater reliability studies for each of the 47 biomarkers. Biomarkers rejected on intra-rater reliability testing are shown in red. Biomarkers rejected on inter-rater reliability testing are shown in blue

Marker	Inter-observer ICC	Intra-observer ICC		
		Edinburgh	Newcastle	London
Neutrophil CD14	1	1	1	1
Neutrophil CD15	1	1	1	1
Neutrophil CD24	1	1	1	1
Neutrophil CD35	1	1	1	0.9999
Neutrophil CD64	1	1	1	1
Neutrophil CD312	1	1	1	0.9995
Monocyte CD14	0.9998	1	1	0.9993
Monocyte CD15	0.9998	1	0.9998	0.9997
Monocyte CD24	0.999	0.9986	0.9996	0.999
Monocyte CD35	0.9992	0.9997	0.9997	0.9994
Monocyte CD64	0.9997	0.9998	0.9996	0.9993
Monocyte CD312	0.9943	0.9998	0.9997	0.9985
HLA-Dr expression on all dendritic cells (DCs)	0.7785	0.9557	0.9675	0.935
Myeloid DC as % of parent cell	0.829	0.8373	0.9942	0.7764
Non-specific DC as % of parent cell	0.864	0.6017	0.9873	0.9364
Plasmacytoid DC as % of parent cell	0.7611	0.9704	0.9796	0.9434
Total DC as % of non-granulocyte cells	0.7484	0.967	0.9582	0.9266
Myeloid DC as % of non-granulocyte cells	0.8208	0.9305	0.9961	0.8775
HLA-Dr expression on all monocytes	0.9994	0.9996	0.9994	0.9997
HLA-Dr expression on classical monocytes	0.9989	0.9997	0.9995	0.9997
HLA-Dr expression on non-classical monocytes	0.9299	0.9762	0.9933	0.9962
HLA-Dr expression on intermediate monocytes	0.9962	0.9976	0.9976	0.989
Classical monocytes, as % of all monocytes	0.929	0.9985	0.9938	0.9928
Non-classical monocytes, as % of all monocytes	0.9744	0.9887	0.9716	0.9907
Intermediate monocytes, as % of all monocytes	0.959	0.9929	0.9673	0.9678
Neutrophil CD11b.mfi	0.9999	1	0.9999	0.9999
Monocyte CD11b.mfi	0.9968	0.9978	0.9991	0.9995
Neutrophil CD16hi	0.7295	0.9843	0.8097	0.915
Neutrophil CD62Lhi/CD16mid	0.798	0.9844	0.802	0.9383
Neutrophil CD62Llow/CD16low	0.9493	0.9962	0.9929	0.9827
CD274 expression on CD8 lymphocytes	0.812	0.5054	0.9903	0.9771
CD274 expression on classical monocytes	0.9997	0.9998	0.9999	0.9453
CD274 expression on non-classical monocytes	0.9855	0.6597	0.997	0.9182
CD274 expression intermediate monocytes	0.9793	0.9919	0.9963	0.9594
CD274 expression on neutrophils	0.9998	0.998	1	0.9949
CD274 expression on all monocyte	0.9997	0.9998	0.9997	0.9997
CD279 expression on CD8 lymphocytes	0.914	0.9937	0.9682	0.9925
CD279 expression on classical monocytes	0.9943	0.975	0.9993	0.9874
CD279 expression on non-classical monocytes	0.1717	-0.1038	0.9924	0.974
CD279 expression on intermediate monocytes	0.9242	0.9189	0.9908	0.9689
CD279 expression on neutrophils	0.9978	0.9977	0.9989	0.9966
CD279 expression on all monocytes	0.9935	0.9703	0.9989	0.9863
NK measured as % of total lymphocytes	0.9985	0.9998	0.9989	0.9963
NKT measured as % of total lymphocytes	0.9883	0.9986	0.9958	0.9979
Cytotoxic NK cells, measured as % of NK cells	0.951	0.9888	0.9602	0.8446
Mature NK cells, measured as % of NK cells	0.8212	0.9765	0.9882	0.916
Naïve NK cells, measured as % of NK cells	0.7978	0.9681	0.986	0.9348

eTable-3: Rationale for Biomarkers selected for discriminant analysis in cross cohort comparison

Biomarker	Biological role	Cross cohort Kruskal Wallis test	Expert assessment	Selection
Neutrophil biomarkers				
CD14	LPS receptor with TLR4	P < 0.0001	Values cross cohorts mainly within 0-100 MFI range so limited variability. Cell numbers high. Differences mainly between cohort 1 and cohorts 2&3 which had similar values and range. Overall values lower in cohort 1.	NO
CD15	Carbohydrate adhesion module	P < 0.0001	Values for MFI range widely to maximum >8000. Cell numbers high. Clear cross cohort differences: highest cohort 3; lowest cohort 2; intermediate cohort 1.	YES
CD24	Cell adhesion glycoprotein; mediates cell apoptosis. Neutrophil expression	P < 0.001	Values for MFI cross cohort range widely to >50000. Cell numbers high. Clear cross cohort differences: highest cohort 1&2 with wide variability; lowest in cohort 1. Potential biological significance	YES
CD35	Complement receptor (type 1).	P < 0.0001	Values for MFI range widely to maximum >60000. Cell numbers high. Clear cross cohort differences: highest cohort 2; lowest cohort 3; intermediate cohort 1.	YES
CD64	Fc-gamma receptor 1	P < 0.0001	Values for MFI range widely to maximum >20000. Cell numbers high. Clear cross cohort differences: highest cohort 2; lowest cohort 3; intermediate cohort 1.	YES
CD312	G-protein coupled molecule	P < 0.0001	Values for MFI range to maximum >3000. Cell numbers high. Clear cross cohort differences: highest cohort 2; lowest cohort 1; intermediate cohort 2.	YES
CD11b	Complement receptor 3	P < 0.0001	Values for MFI range widely to maximum >60000. Cell numbers high. Clear cross cohort differences: highest cohort 1; lower and similar in cohorts 2&3.	YES
CD62L low CD16 low as % total neutrophils	Exploratory group	P < 0.0001	Values for percent mostly <2% with small number of outliers. Percent in small numbers ranged to >5%. Cell numbers very low. Cross cohort differences lack potential for discrimination.	NO
CD274	PD1 ligand	P < 0.0001	Values for MFI range to maximum >1000. Cell numbers high. Clear cross cohort differences: highest cohorts 1&2; lowest 3.	YES
CD279	PD1	P < 0.0001	Values for MFI range to maximum ≈1000. Cell numbers high. Clear cross cohort differences: wide range of values in cohorts 1&2 with lower values than cohort 3. Values consistently high in cohort 3.	YES
Monocyte biomarkers				
CD14	LPS receptor with TLR4	P = 0.277	No cross cohort differences. Biomarker was mainly selected as monocyte selection marker	NO

CD15	Carbohydrate adhesion module	P = 0.036	Minimal cross cohort differences	NO
CD24	Cell adhesion glycoprotein; mediates cell apoptosis. Neutrophil expression	P < 0.017	Minimal cross cohort differences. Lacks biological plausibility for monocytes.	NO
CD35	Complement receptor (type 1).	P < 0.0001	Values for MFI range widely to maximum >40000. Cell numbers moderate. Clear cross cohort differences: highest cohorts 1&2; lowest cohort 3.	YES
CD64	Fc-gamma receptor 1	P < 0.0001	Values for MFI range widely to maximum >100000 (in cohort 1). Cell numbers intermediate. Clear cross cohort differences: highest cohorts 1&2; lowest cohort 3.	YES
CD312	EMR2 cell surface marker on monocytes	P = 0.009	Values for MFI range to maximum >6000. Cell numbers intermediate. Some cross-cohort differences: highest cohort 2; lower in cohorts 1&3.	YES
CD11b	Complement receptor 3	P < 0.0001	Values for MFI range widely to maximum >80000. Cell numbers intermediate. Some cross cohort differences: highest cohorts 1&2; lower cohorts 3.	YES
CD274 total monocytes	PD1 ligand - check point inhibitors family	P < 0.0001	Values for MFI range to maximum >1000. Cell numbers intermediate. Some cross cohort differences: highest cohorts 1&2; lowest 3.	YES
CD279 total monocytes	PD1 – check point inhibitors family	P < 0.0001	Values for MFI range to maximum ≈500. Cell numbers intermediate. Clear cross cohort differences: wide range of values in cohorts 1&2 with lower values than cohort 3. Values consistently high in cohort 3.	YES
HLA-DR total monocytes	Antigen presentation	P < 0.0001	Values for MFI range to >30000. Cell numbers high. Clear cross cohort differences with highest levels in cohort 3, lowest in cohort 2, and intermediate with wide range in cohort 1.	YES
Percent classical monocytes		P<0.0003	Although differences apparent across the three groups, the cell numbers for non-classical and intermediate monocyte groups low. General patterns suggest lower percentages of classical monocytes in cohort 2 relative to cohorts 1&3; lower percent non-classical monocytes in cohort 1 compared to cohorts 2&3; lower percent of intermediate monocytes in cohort 3 compared to cohorts 1&2. Likely mathematical linkage and collinearity between these biomarkers, which would be problematic in discriminant and multivariable analysis.	NO
Percent non-classical monocytes		P < 0.0001		
Percent intermediate monocytes		P < 0.0001		
CD274 on classical monocytes	PD1 ligand - check point inhibitors family	P < 0.0001	Although differences across groups exist, cell numbers small and absolute differences in MFI values between groups small. Likely mathematical linkage and collinearity with CD274 on total monocytes, which would be problematic in discriminant and multivariable analysis	NO
CD274 on intermediate monocytes	PD1 ligand - check point inhibitors family	P < 0.0001		
CD279 on classical monocytes	PD1 – check point inhibitors family	P < 0.0001	Although differences across groups exist, cell numbers small and absolute differences in MFI values between groups small. Likely mathematical linkage and collinearity with CD274 on total monocytes, which would be problematic in discriminant and multivariable analysis	NO
CD279 on intermediate monocytes	PD1 – check point inhibitors family	P < 0.0001		
HLA-DR on classical monocytes		P < 0.0001	Patterns all mirror differences across cohorts for HLA-DR on total monocytes. Cell numbers for non-classical and intermediate monocytes very low. Likely mathematical linkage and collinearity with HLA-DR on total monocytes, which would be problematic in discriminant and multivariable analysis	NO
HLA-DR on non-classical monocytes		P < 0.0001		

HLA-DR on intermediate monocytes		P < 0.0001		
Lymphocyte biomarkers				
CD279 on CD8 T cells	PD1 – check point inhibitors family	P < 0.0001	Cell numbers intermediate. Some cross cohort differences: highest cohorts 1 and 2.	YES

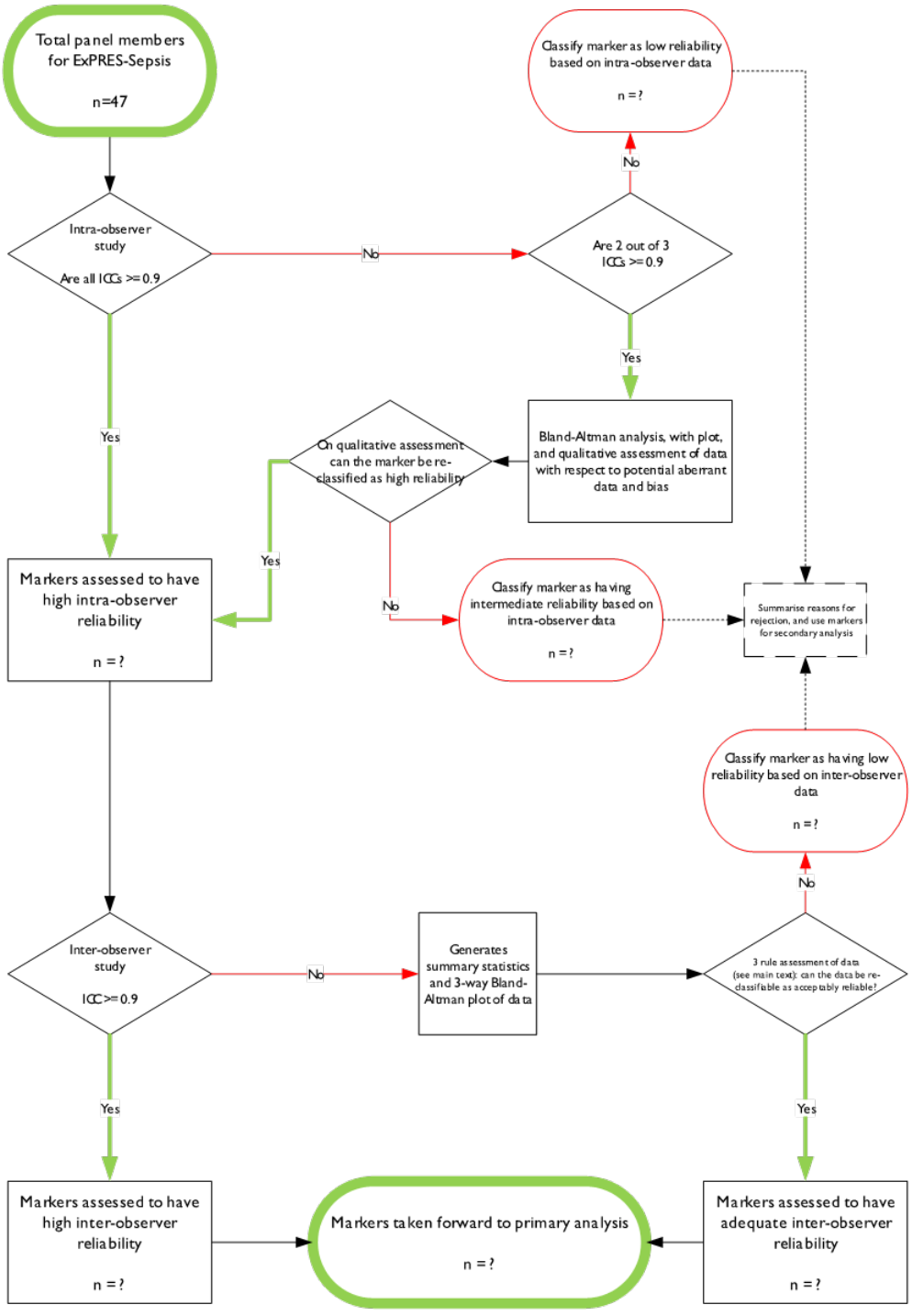
eTable-4: Members of the independent expert review group who reviewed the provisional data from reliability, cross cohort comparisons, and discriminant analysis for the primary and secondary outcomes. This group recommended the post hoc extreme phenotype analysis to further explore differences between patients who subsequently recovered quickly versus progressed to severe sepsis.

Name	**Position, Institution**
Mervyn Singer	Professor of Intensive Care Medicine, University College, London, UK
Jean-Daniel Chiche	Professor of Critical Care Medicine, Hospital Cochin, Paris, France
Paul Dark	Professor of Critical Care Medicine, University of Manchester, Manchester, UK

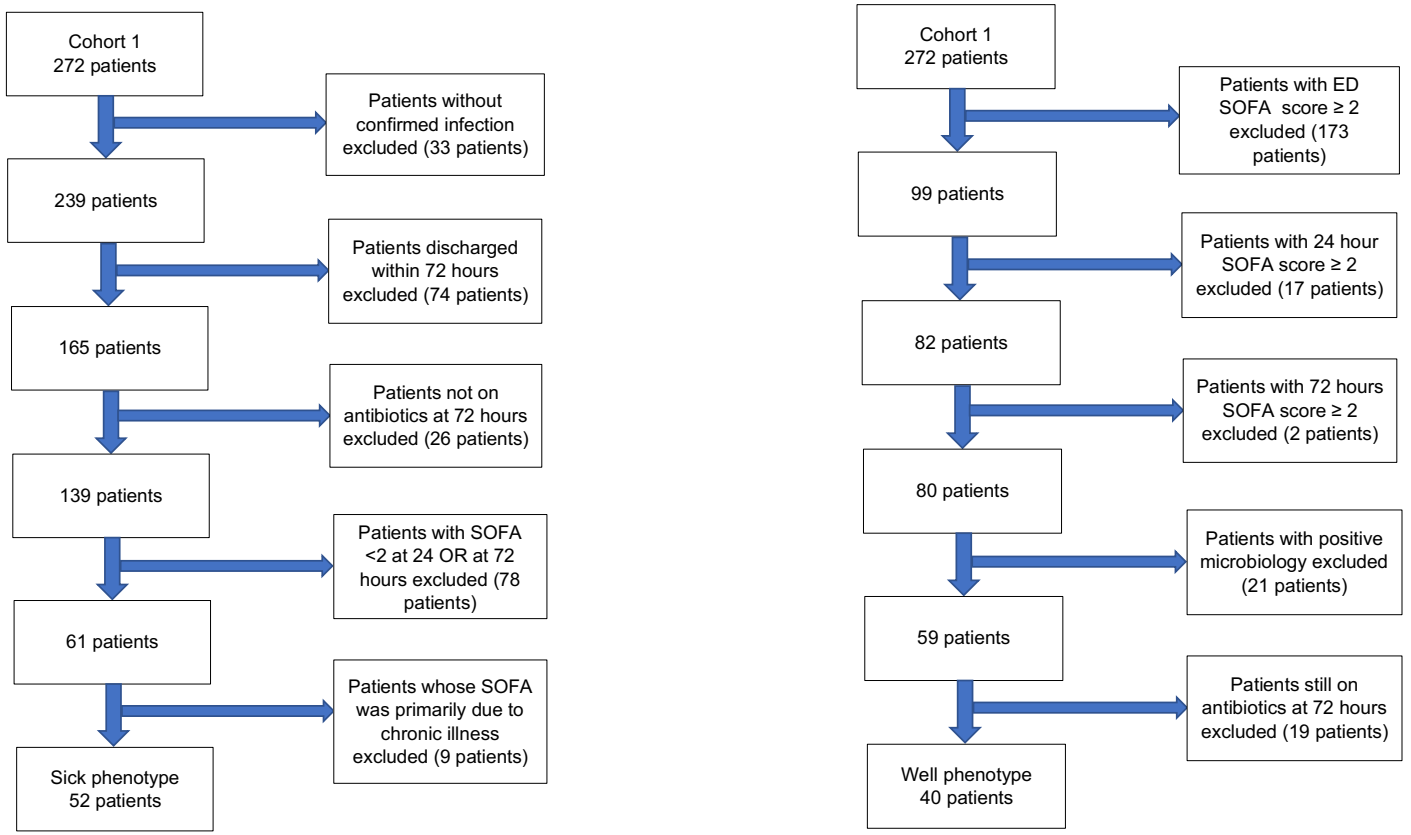
eTable-5: Comparison of recent leukocyte biomarker studies using multi-site flow cytometry with standardisation for illness trajectory prediction [2-4]

Study characteristics	Current study (EXPRESS) N=259	Guerin E at al [4] N=177	Daix T el al[2] N=781	Conway-Morris A et al [3] N=138
Primary objective	Predict deterioration to develop sepsis sepsis-3 sepsis within 24 or 72 hours	Predict early evolution (deterioration or stability/improvement) of sepsis at 48 hours	Predict early evolution (deterioration or stability/improvement) of sepsis	Validate[6] cellular markers of immune dysfunction to stratify risk of secondary infection
Case definition at sampling	Patients with suspected infection attending ED	Patients with sepsis, severe sepsis or septic shock	Patients with sepsis, severe sepsis or septic shock	Critically ill patients predicted to remain in ICU for >=48 hours
Sites (N)	4	1	11	4
Number of leukocyte biomarkers evaluated	47 leukocyte biomarkers including leukocyte subsets (see eTable-1) assessed for reliability, discriminant value, followed by best subsets logistic regression	24 markers and 23 leukocyte subsets CD36; CD2; CD294; CD19; CD16; CD45; CD11b; CD16; CD8; CD64; CD11c; CD10; CD24; CD34; CD123; CD138; CD4; CD38; CD25; CD56; CD127; CD3; CD116; HLA-DR	CD64; CD10; CD3; CD24; CD11b; CD16; CD45	Neutrophil Cd88; Monocyte HLA-DR; proportion of regulatory T cell subsets
Key findings	Optimum biomarker combination of increased neutrophil CD24 and neutrophil CD279, and reduced monocyte HLA-DR expression to predict subsequent deterioration to sepsis	Immature granulocytes (CD10dim CD16dim) predicted clinical deterioration	Immature granulocytes associated with clinical worsening, when associated with T cell lymphopenia	Confirmed our previous findings[6]
Comparison of key findings of other studies with our EXPRESS study		CD16low subset did not have cross cohort discrimination in our EXPRESS study	CD64 MFI had univariate association, which disappeared with best-subsets logistic regression. Lymphopenia did not have cross cohort discrimination; CD24 expression in neutrophils was associated with clinical deterioration in our EXPRESS study	Different study population; HLA-DR was associated with clinical deterioration to sepsis in our EXPRESS study
Biological relevance of key markers reported in each study	CD24 expressed on mature granulocytes and B cells; down-regulated on neutrophils in sepsis, induces neutrophil apoptosis which is impaired in sepsis. CD279 and HLA-DR are markers of associated with sepsis related immunosuppression consistently reported in literature	Myeloid derived immature granulocytes appear cytotoxic towards T lymphocytes	CD64 is a Fc gamma receptor expressed on leukocytes; consistently reported as a diagnostic marker for sepsis	

eFigure-1: Flow diagram showing the decision analysis for assessing intra- and inter-rater reliability for the 47 biomarkers, and selecting biomarkers considered reliable for evaluation in cross cohort comparisons. This algorithm is replicated as presented in the protocol manuscript [1].

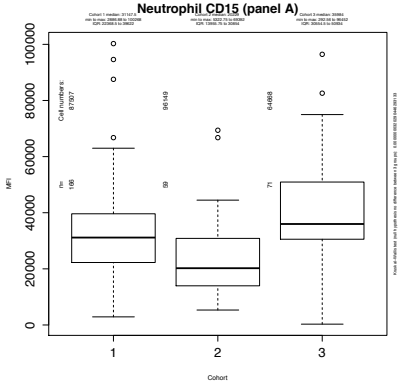


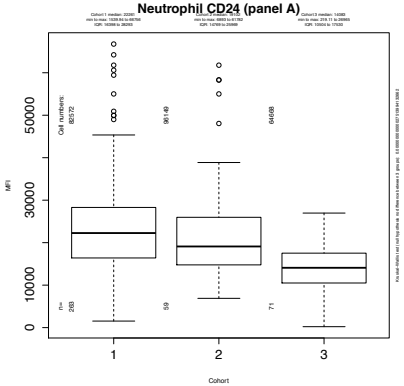
eFigure-2: Extreme phenotype derivation

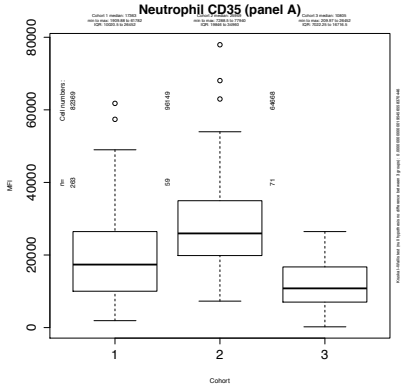


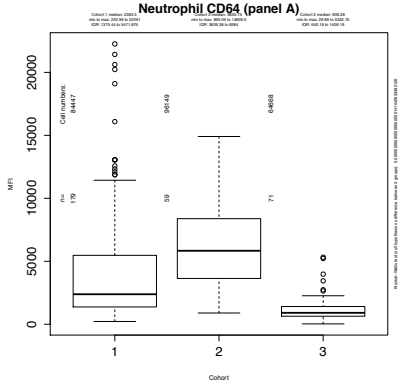
eFIgure-3: Cross cohort comparison of significant markers taken forward for further evaluation

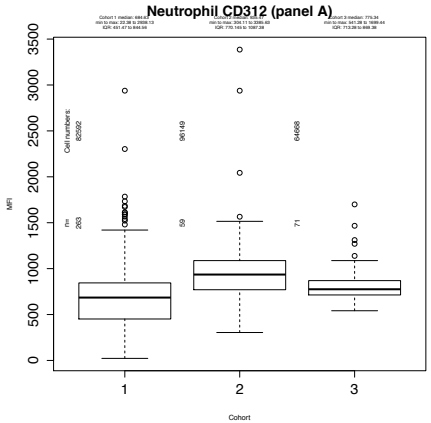
eFIgure-3a: Neutrophil markers

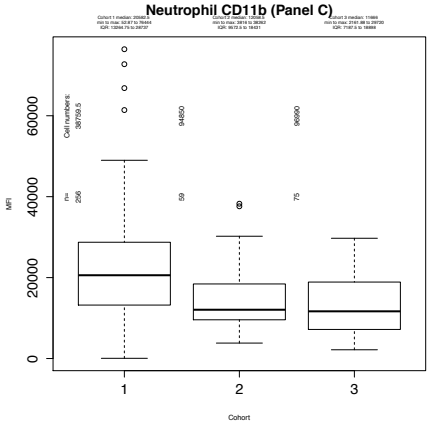


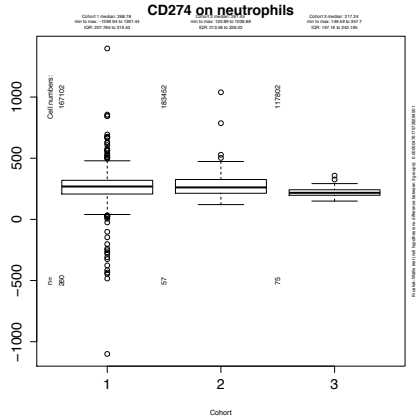


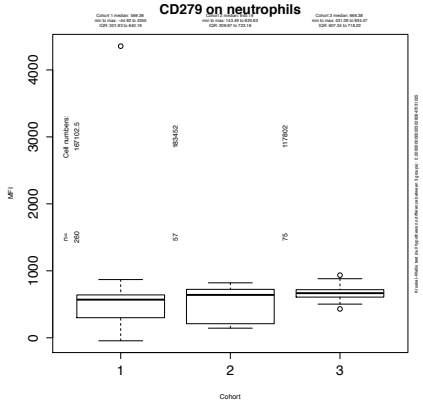






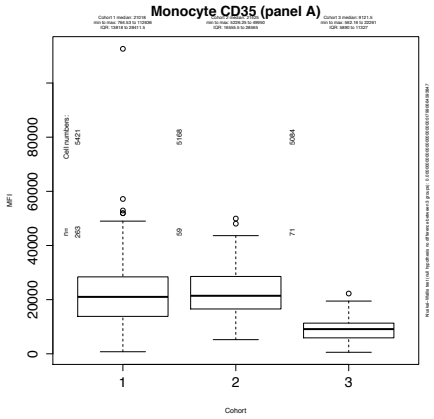


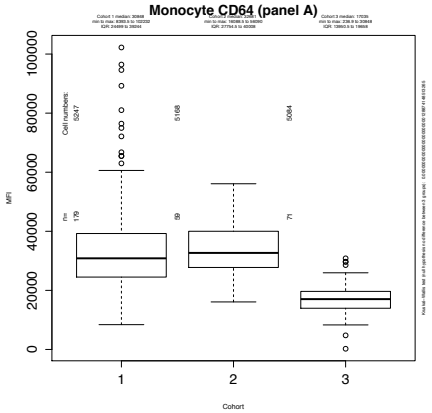


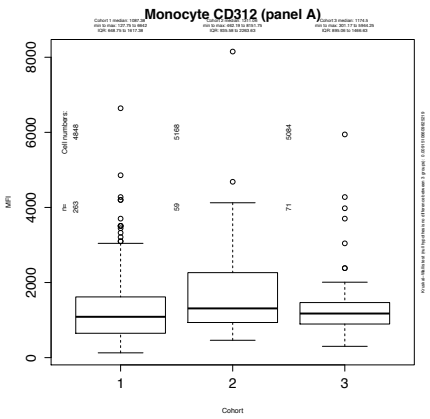


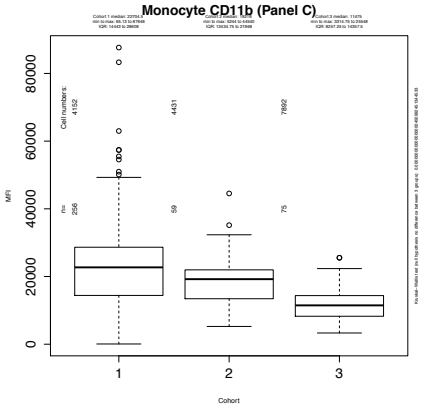
eFIgure-3: Cross cohort comparison of significant markers taken forward for further evaluation

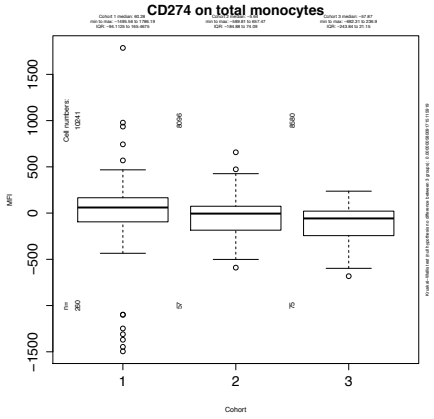
eFIgure-3b: monocyte biomarkers and CD279 on CD8-T cells

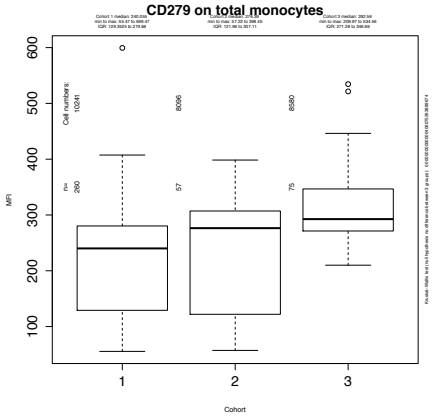


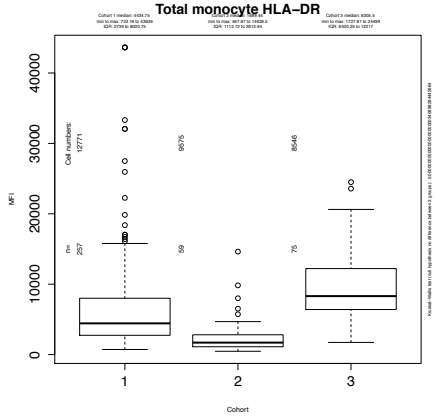


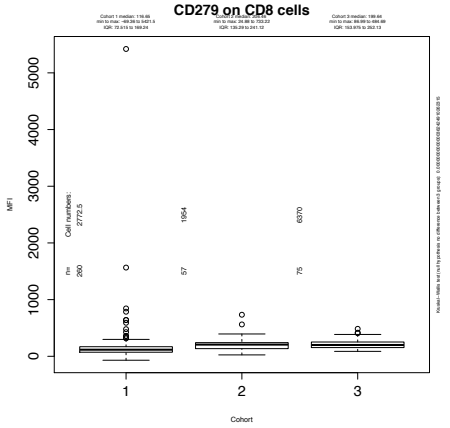




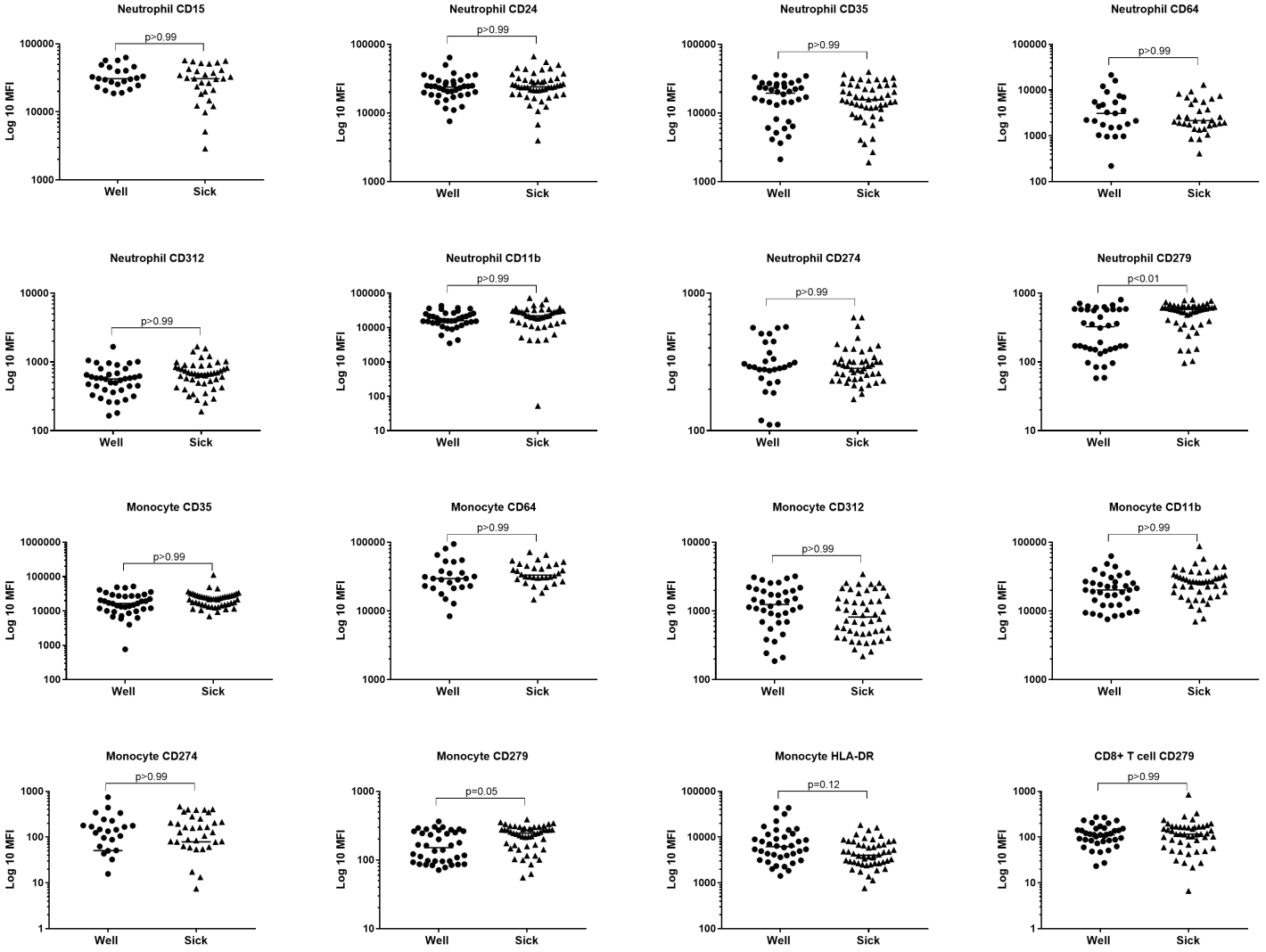








eFigure-4: Biomarker profile differences in the well versus sick extreme phenotype



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1. Datta D, Conway Morris A, Antonelli J, et al. Early PREdiction of Severe Sepsis (ExPRES-Sepsis) study: protocol for an observational derivation study to discover potential leucocyte cell surface biomarkers. BMJ Open. 2016;6(8):e011335.
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3. Conway Morris A, Datta D, Shankar-Hari M, et al. Cell-surface signatures of immune dysfunction risk-stratify critically ill patients: INFECT study. Intensive Care Med. 2018;44(5):627-635.
4. Guerin E, Orabona M, Raquil MA, et al. Circulating immature granulocytes with T-cell killing functions predict sepsis deterioration*. Crit Care Med. 2014;42(9):2007-2018.
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6. Conway Morris A, Anderson N, Brittan M, et al. Combined dysfunctions of immune cells predict nosocomial infection in critically ill patients. Br J Anaesth. 2013;111(5):778-787.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Manuscript reference; page number
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	**Title; Page-1**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	**Abstract; Page-5 and Page-6**
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	**Introduction; Page-7 and Page-8**
Objectives	3	State specific objectives, including any prespecified hypotheses	**Introduction Page-7 and Page-8; Parah-3**
Methods			
Study design	4	Present key elements of study design early in the paper	**Methods: Page-8, 9, 10, 11, 12, and Page-13 Electronic supplement; Page 2, 3, 4, 5, 6, 7 and Page-8**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	**Methods: Page-8, 9, 10, 11, 12, and Page-13 Electronic supplement**
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	**Methods: Page-8 Online only supplement; Page-2,3,**
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	**Not applicable**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	**Methods: Page-8, 9, 10, 11, 12, and Page-13 Online only supplement; Page-4,5, 6, 7 and Page-8 Statistics Page-6**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	***n/a***
Bias	9	Describe any efforts to address potential sources of bias	**Discussion; Page 18**
Study size	10	Explain how the study size was arrived at	**Methods Page 10**
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	**Statistics; Page 10, 11, 12 and Page-13**
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	**Statistics; Page 10, 11, 12 and Page-13**
		(*b*) Describe any methods used to examine subgroups and interactions	**Statistics; Page 10, 11, 12 and Page-13**
		(*c*) Explain how missing data were addressed	**Statistics; Page 10, 11, 12 and Page-13**
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed	**Statistics; Page 10, 11, 12 and Page-13**
		(*e*) Describe any sensitivity analyses	**N/a**

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	**Page-13 and Page-14**
		(b) Give reasons for non-participation at each stage	**n/a**
		(c) Consider use of a flow diagram	**n/a**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	**Table-1**
		(b) Indicate number of participants with missing data for each variable of interest	**As above**
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	**Table-1**
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	**Table-1; Figure-1; eTable-2; Table-3**
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	**Table-3**
		(*b*) Report category boundaries when continuous variables were categorized	**Table-3**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	**AUROC and Odds ration with each SD increase in biomarker value**
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	**n/a**
Discussion			
Key results	18	Summarise key results with reference to study objectives	**Page 16; Parah-2**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	**Page 18**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	**Discussion: Pages 16, 17, 18, and Page-19**
Generalisability	21	Discuss the generalisability (external validity) of the study results	**Discussion: Page Page-16 paragraph-; Page-19**
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	**Page-3**

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies. **Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.